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1 **Toward a comprehensive study for multielemental quantitative LA-ICP MS**
2 **bioimaging in soft tissues**

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16

17 **Abstract**

18 Quantitative localization of metals in biological tissue sections is critical to obtain insight into
19 metal toxicity mechanisms or their beneficial characteristics. This study presents the
20 development of a quantitative LA-ICP MS bioimaging methodology based on the polymer film
21 strategy and internal standardization. To maximize the number of elements mapped, an
22 aqueous soluble polymer (dextran) was selected. Among the elements studied, the great
23 majority (eight out eleven), i.e., Co, Ni, Cu, Zn, Se, Mo, Cd and Pt, exhibited linear regression
24 after LA-ICP MS analysis of metal-spiked polymer standards. Methodology performances were
25 carefully assessed as a function of the three internal standards (In, Rh and Ir) considered, the
26 analytical operational conditions (ICP power, addition of O₂ to ICP, and laser fluency) and the
27 thickness of the biological tissue section. The results indicated that three groups (Co, Mo; Ni,
28 Cu, Pt; and Zn, Se, Cd) of elements could be distinguished from their analytical response as a
29 function of analytical conditions and the internal standard. These different element behaviors

30 appeared to be mainly First Ionization Potential dependent (FIP). For elements with lower FIP
31 (Co, Ni, Cu, Mo and Pt), differential responses due to carbon load in the ICP MS plasma could
32 be efficiently corrected as a function of analytical conditions. Matrix effects were more
33 pronounced for higher FIP elements (i.e., Zn, Cd and Se), and analysis of <10- μ m thin sections
34 without the addition of O₂ to ICP MS plasma is recommended. LODs are in the range of 0.1-
35 0.5 μ g g⁻¹ for Co, Mo, Cu, Ni, Pt and Cd as well as 0.9 and 1 μ g g⁻¹ for Zn and Se, respectively.
36 The methodology was validated by means of a homemade metal-spiked kidney homogenate
37 analyzed by LA-ICP MS imaging, and Co, Ni, Cu, Mo, and Pt provided the closest concentrations
38 (5-29% bias) to the target values determined by ICP MS after mineralization. The methodology
39 was applied to two types of clinical human samples undergoing different sample preparation
40 protocols that did not affect internal standard homogeneity in the polymer film. This
41 methodology is the first reported for the quantitative bioimaging of eight elements
42 simultaneously.

43

44 **Keywords:** LA-ICP MS, bioimaging, quantification, multielemental, polymer films

45

46 **Introduction**

47 Metals affect many aspects of life. Some metals are essential or beneficial, whereas others
48 are deleterious or even toxic (i.e., iron is the most widely distributed metal and is essential to
49 oxygen transport; mercury is neurotoxic, but its presence with selenium inhibits its toxicity)
50 [1-2]. The bioinorganic chemistry of the organism/cell governs the role of each metal, stressing
51 the requirement for a fine description of metal localization. Thus, visualizing metals
52 distribution at the organism and cellular level is critical for understanding how metals function
53 and how their functions might be altered [3]. Moreover, the measurement of the
54 concentration of metal in a specific area of a biological tissue is of paramount importance.
55 Indeed, metal deficiency or overload can be a proof of disease (i.e., iron misbalance in
56 neurological diseases [4-5], high copper levels in sera of patients with breast cancer [6]), and
57 studies of metal-based drug penetration into tumor facilitates treatment protocol
58 development [7-9].

59 LA-ICP MS is the most advanced technique for metal species quantification given its
60 sensitivity (low μ g g⁻¹ level) [10-13] and sufficient spatial resolution (μ m and sub- μ m).

61 Nevertheless, quantification of metals in tissue thin sections using LA-ICP MS is problematic.
62 Indeed, the heterogeneity of biological samples and the lack of suitable standard material give
63 rise to difficulties (ablation, transport, ionization into ICP source, signal drift over analysis time
64 and matrix effects) that are well documented [14]. Various approaches have been proposed
65 to improve metal quantification by LA-ICP MS in biological tissues; however, there are still
66 discrepancies regarding a truly universal calibration and quantification protocol [14-15].

67 The quantification methodologies by LA-ICP MS can be classified into three strategies. One
68 of the pioneer works corrected instrumental or elemental sensitivity through the addition of
69 an internal standard into either the ablation cell or the ICP source [16]. However, correction
70 strategies for ablated material variation and transport are lacking. External calibration
71 strategies have emerged using matrix matched standards, i.e., gelatin-dried droplets [13] and
72 standards [17], embedding resins [18], or a piece of the sample itself [19-20] spiked with
73 different amounts of metal. These strategies that lack internal standard require the complete
74 ablation of the standard as well as the sample itself, and the lack of a blank may cause issues
75 with the measurement of endogenous elements, such as Cu, Zn, and Fe, among others.
76 Internal standardization strategies consist using either a native element homogeneously
77 distributed in the sample [16], but this is often difficult to find, or by introducing an internal
78 standard by full immersion [21] of the slice sample into the metallic solution, but this method
79 potentially bears the risk of metal redistribution. Other internal standardization strategies rely
80 upon adding an internal standard spiked layer either at the top or below the sample. Among
81 these strategies, printed metal spike ink [22] onto the sample surface, spraying an internal
82 standard coating on top of the sample [23] and depositing the internal standard between the
83 sample and the substrate [24] represent the main strategies being developed. Here, the
84 complete ablation of the sample and internal standard ensures their simultaneous transport.
85 Internal standard-spiked ink methods may be limited when endogenous elements, such as
86 copper, are under study given their natural presence in ink. Coating the sample with an
87 internal standard spray is typically performed by sputter using Au, Cr, Mo, Ni, Ti, W, Ag, Pt, Cu,
88 Pd, Co, or Fe as commercially available metal targets. Therefore, given that most of these
89 metals may be encountered in biological samples, applications are often limited to the use of
90 Au as internal standard, which is only appropriate for a set of elements. Interfacing a metal
91 spiked polymer film between the support (glass slide) and the sample requires some sample
92 preparation similar to that noted with others methodologies and the use of a spin-coater, but

93 leaves free ourselves of the choice of the polymer and the internal standard according to the
94 bioimaging application. Autin et al [24] has proposed an organic soluble (xylene and
95 chlorobenzene) polymer (polymethylmethacrylate) requiring organometallic standards (Cu
96 and Zn for calibration and Y and Ru as internal standards) for spiking experiments.
97 Normalization with Y and Ru improved the accuracy of Cu and Zn quantification on a 20- μ m
98 tissue slice despite increased standard deviation compared to ^{13}C normalization. This
99 methodology is very promising given that it can be potentially used in a wide panel of various
100 applications (different elements to map). Nevertheless, the use of such a type of polymer is a
101 limiting parameter in the choice of internal standards and the handling of organometallic
102 standards (non-aqueous soluble); thus, further developments should be considered.

103 The choice of the internal standard for an effective normalization of the analyte is of
104 course of paramount importance. The use of carbon (^{13}C) has been long time discussed to
105 correct for variation in ablated mass, but its low sensitivity (11.6 eV first ionization potential
106 (FIP)) and its heterogeneous distribution in biological samples prevent its use for quantitative
107 bioimaging by LA-ICPMS. The choice of an isotopic variant of the analyte itself would be ideal
108 to correct for instrumental signal drift, matrix effects or analyte losses [25-26]. However, this
109 is not always possible (case of monoisotopic element) and given that this method would
110 ideally imply the mixing of the enriched isotope with the sample at the earliest point in the
111 preparation procedure, this would limit the field of applications (impossible in case of clinical
112 samples for example). The selection of the analyte/internal standard pair is typically made
113 based on the criteria of a close mass and first ionization potential between the two elements.
114 Autin et al [27] recommended the incorporation of an internal standard with an atomic mass
115 close to the analyte for imaging experiments to correct for variation in ablated mass and
116 sampling cell position. In addition, ^{197}Au , which has a high mass and mid-range ionization
117 potential (9.22 eV), compensates for instrumental drifts, matrix-related ablation differences
118 and day-to-day signal changes for ^{63}Cu (FIP 7.73 eV) and ^{64}Zn (9.39 eV) analysis despite the
119 high mass difference [23]. Therefore, the choice of an internal standard relies on the
120 application.

121 The aim of our study was to develop an internal calibration methodology based on polymer
122 film standards to extend this methodology to the quantitative bioimaging of a wider range of
123 elements by LA-ICP MS. Thus, the nature of the polymer was first considered by targeting
124 aqueous soluble polymers for easier spiking with metals. The methodology aimed at the

125 application to a set of elements encountered in biological samples given their natural
126 occurrence, their therapeutic use or their presence as pollutants. Several internal standards
127 were investigated for normalization of the various elements for increased stability and
128 sensitivity. The accuracy of our methodology was assessed by means of a homemade matrix-
129 matched standard at three concentrations levels and different thin-section thicknesses. The
130 influence of experimental conditions of LA-ICP MS analysis, such as ICP power and
131 composition of ICP plasma (O_2 addition), were also evaluated to minimize differential analyte
132 responses over the imaging run time.

133

134 **Materials and methods**

135 ***Reagents and samples***

136 Elemental solutions at 50 000 mg L⁻¹ Fe, Cu and Zn and 10 000 mg L⁻¹ of Pt, P, Co, Ni, Se, Mo,
137 Hg, and Cd were purchased from SCP Science (PlasmaCAL, Courtaboeuf, France). Briefly, 1 000
138 mg L⁻¹ solutions of In, Ir and Rh used as internal standards were purchased from SCP Science.
139 Intermediate elemental solutions at 100 and 2500 mg L⁻¹ were prepared in 2% ultrapure HNO₃
140 (70% INSTRA-ANALYZED, Baker, Illkirch-Graffenstaden, France). Dextran polymer (Mw of ca.
141 500 000 g mol⁻¹) was purchased from VWR (Fontenay-sous-Bois, France). All solutions were
142 prepared with milliQ water (resistivity $\geq 18.2 \Omega \text{ cm}$) obtained from a Milli-Q Elix water
143 purification system (Millipore, Molsheim, France).

144

145 ***Polymeric film preparation and thickness characterization***

146 Here, 60 mg mL⁻¹ dextran solution was prepared in water by simple vortexing. A portion of
147 this solution was spiked with the internal standards solution (In, Rh, Ir) to obtain a theoretical
148 concentration of 10 $\mu\text{g g}^{-1}$ before preconcentration by spin-coating and dedicated as sample
149 deposit substrates after spin-coating. The other portion of the solution was spiked with
150 internal standard solution and different amounts of metal solutions (Fe, Cu, Zn, Pt, P, Co, Ni,
151 Se, Mo, Hg, Cd) to produce calibrants for quantification by LA-ICP MS after spin-coating. In
152 each standard, two ranges of theoretical elemental concentrations were aimed in polymeric
153 film, i.e., from 0 to 80 $\mu\text{g g}^{-1}$ for minor elements (Co, Ni, Se, Mo, Cd and Hg) and from 0 to 400
154 $\mu\text{g g}^{-1}$ for major elements (P, Fe, Cu, Zn and Pt) while maintaining the same internal standard
155 concentration (i.e., 10 $\mu\text{g g}^{-1}$) as for samples deposit substrates. For sample deposit substrates

156 and each calibration solution, the proportion of polymer remained constant, whereas final
157 volume was adjusted with milli-Q water. Each solution was individually spin-coated as follows.
158 Approximately 600 μL of each 45- μm filtrated (45 μm cellulose filter, VWR, France) solution
159 was loaded onto the surface of a cleaned 2.6 x 2.6 cm glass slide (VWR international, Fontenay-
160 sous-bois, France) and spin-coated (Polos SPIN150i spin-coater model, SPS, Vourles, France)
161 following optimized conditions at 4500 rpm for 60 s under a light N_2 gas stream. After spin-
162 coating, film substrates were ready for analysis or sample deposit. The average mass ($n=5$) of
163 polymeric film was determined for each calibration point using an ultrahigh precision balance
164 (Automated-S microbalance XP6, Mettler Toledo, Viroflay, France) by weighting glass slide
165 before and after film deposition (providing a clean glass slide surface). Film thickness was
166 determined by optical profilometry using a WYKO NT1100 Optical profiling System (Veeco,
167 Dourdan, France) in Vertical Shift Interference (VSI) mode after using a scalpel to scratch the
168 polymeric film on the glass substrate and coating with a thin Au layer (1-5 nm) for 30 s to make
169 the surface reflective to light.

170 ***Preparation of matrix-matched tissue standards***

171 A piece of pig kidney was purchased at a local supermarket and was homogenized using an
172 Ultraturax (IKA T10 basic, Imlab, Lille, France). Aliquots of homogenized kidney were used as
173 control standard, 2.5 $\mu\text{g g}^{-1}$ spiked standard, 50 $\mu\text{g g}^{-1}$ spiked standard and 250 $\mu\text{g g}^{-1}$ spiked
174 standard (concentrations expressed as major elements). To prepare these standards,
175 approximately 5 g of homogenized kidney tissue was placed into a 10-mL tube, nonspiked (for
176 control standard), or spiked with an appropriate volume of 100 $\mu\text{g g}^{-1}$ (for 2.5 $\mu\text{g g}^{-1}$ standard)
177 and 2500 $\mu\text{g g}^{-1}$ (for 50 and 250 $\mu\text{g g}^{-1}$ standard) of intermediate elemental solutions before
178 homogenization with Ultraturax. For each condition, 2 aliquots were transferred (as compact
179 as possible to form a cylinder) into 2-mL Eppendorf tubes, frozen under liquid N_2 and stored
180 at -18°C until cryomicrotomy.

181 ***Cryomicrotomy***

182 ***Matrix-matched tissue standard-*** Homogenate kidney standards were removed from
183 Eppendorf tube and embedded with OCT (Q Path[®] VWR). Thin sections of 8, 10, 14, 30 μm
184 thickness were produced from the kidney standards using a cryomicrotome (CM 1950 model,
185 Leica, France) where -18°C and -15°C temperatures were set for the specimen and the
186 chamber, respectively, and deposited on the polymeric substrate.

187 **Patient tissues-** For sanitary, safety and regulatory issues, human tissue samples (ovary and
188 tumoral nodules) were collected, frozen at -80°C and processed at the Institut du Cancer de
189 Montpellier in accordance with standard practices. Here, 8-µm thin sections of ovary were
190 obtained from frozen tissues using a Microm HM 550 (Thermo Scientific) cryotome set at -
191 18°C and -20°C for specimen and chamber, respectively. Thin frozen sections were deposited
192 on top of the polymeric film substrate and allowed to dry under vacuum. Tumor tissues were
193 fixed with formaldehyde and paraffin-embedded prior to microtomy (MICROM HM 340 E,
194 Thermo Scientific) sectioning at 8 µm. Paraffin-embedded sections were transferred into a
195 heated (40°C) water bath to avoid shrinkage, and the polymeric film substrate was immersed
196 (5 s) in the bath to recover the slice. Samples were then analyzed by LA-ICP MS without any
197 further processing. The storage in a box of such a sample did not exceed 10 days. All patients
198 provided written informed consent before the procedure, and the study was approved in the
199 institutional review board in accordance with the ethical standards of the Helsinki Declaration
200 of 1975.

201

202 **Determination of elemental content by ICP MS**

203 **Kidney standards** - The frozen kidney standard in Eppendorf tube was cut in six 2-3 mm
204 sections (approximately 150 mg each) to determine elemental concentrations in fresh and
205 dried kidney. Half of the sections were quickly frozen under liquid N₂ and placed into a freeze-
206 drying system until complete dryness (water content of ca. 75%). Then, the dried half and the
207 other half of the fresh kidney section were mineralized using 400 µl 69% HNO₃ and heated at
208 80°C in the oven for 265 min. Then, digests were accordingly diluted with milli-Q water to
209 reduce the HNO₃ content to 2% before analysis by ICP MS.

210 **Polymeric films** – For each concentration of metal-spiked polymeric films (calibrants for LA-
211 ICP MS), over 10 substrates, 5 substrates (glass slide + polymer film) (n=4/condition and 5
212 additional blanks (glass slides)) were individually placed into a plastic flask containing 5 mL of
213 2% HNO₃ solution overnight to allow for quantitative polymer film solubilization and metals
214 released into the acid solution before ICP MS analysis. Each left substrate per condition was
215 used as a control for LA-ICP MS analysis. Glass slides were washed into two successive 2%
216 HNO₃ baths to ensure complete metals release into solution. Consecutively, glass slides were
217 dried under a laminar hood, and the absence of metal spiked at the glass slide surface was
218 verified by running several transects (n= 3) by LA-ICP MS analysis over the glass slide surface.

219 The polymeric films of the 10 substrates were previously weighted using the high-precision
220 balance as described above.

221 **ICP MS analysis** was performed with an Agilent 7700x model (Agilent Technologies, Tokyo,
222 Japan). The instrument was tuned daily using a 1 $\mu\text{g L}^{-1}$ solution containing Y, Li, Tl, Ce, and Ba
223 in 2% HNO_3 . ICP MS was operated using a micromist nebulizer, Ni cones and under octopole
224 reaction cell with He gas to lower interferences on ^{31}P , ^{63}Cu , ^{65}Cu , ^{64}Zn , ^{57}Fe , ^{78}Se and
225 ^{80}Se . Element quantification in solution was performed with internal calibration in the range
226 0 to 50 $\mu\text{g L}^{-1}$ for major elements (0-10 $\mu\text{g L}^{-1}$ for minor), and standard addition was performed
227 for element quantification in kidney samples and polymeric films.

228 **LA-ICP MS imaging experiment**

229 A 7700s ICP MS detector (Agilent Technologies, Tokyo, Japan) mounted with platinum cones
230 and S-lenses was used. Preliminary optimization of detection parameters was conducted
231 under the liquid sample introduction mode by means of the 1 $\mu\text{g L}^{-1}$ Y, Li, Tl, Ce, 2% HNO_3
232 tuning solution to ensure maximum sensitivity and optimal mass calibration. A laser ablation
233 system (NWR213, ESI, Fremont, CA, USA) was then coupled to the ICP MS. The ablated
234 particles of sample were transported from the TV2 ablation cell with 800 mL min^{-1} of He mixed
235 by means of a T-piece with Ar gas (ca 1.1 mL min^{-1}) prior to introduction to the dry ICP source.
236 Under coupling mode, carrier gas and ionic lenses voltages were finely tuned using a NIST 612
237 glass standard (NIST, Washington, USA) for a U/Th moving closer to the unit and to maximize
238 sensitivity. ICP MS was used in collision cell mode with He (11 mL min^{-1}) as the
239 collision/reaction gas to allow for monitoring of ^{13}C , ^{31}P , ^{57}Fe , ^{59}Co , ^{60}Ni , ^{63}Cu , ^{64}Zn , ^{65}Cu , ^{66}Zn ,
240 ^{78}Se , ^{80}Se , ^{98}Mo , ^{103}Rh , ^{111}Cd , ^{115}In , ^{193}Ir , ^{194}Pt , ^{195}Pt , ^{200}Hg and ^{202}Hg with 0.08 s integration
241 time except for ^{13}C (0.01 s), yielding a sampling time of 1.567 s.

242 Imaging experiments were performed by sampling of subsequent line scans defined over the
243 sample surface (metal-spiked film standards and biological tissues (kidney, ovary and tumoral
244 tissue) and operating at 20 Hz laser shot repetition rate. For kidney standards and ovary
245 samples, a laser beam size of 75 x 75 μm , a fluency of 3.5 J cm^{-2} , a scan speed of 45 $\mu\text{m s}^{-1}$ per
246 duty cycle of ICP MS acquisition and a 125 μm distance between each consecutive line were
247 used to obtain the desired spatial resolution and signal intensity. For smaller sized samples,
248 such as tumoral tissue (ca 0.5-0.7 cm^2), these parameters were 50 x 50 μm , 4.2 J cm^{-2} , 35 μm
249 s^{-1} and 100 μm , respectively. For each sample line scanned, an ICP MS acquisition file was
250 generated under a csv format.

251

252 **LA-ICP MS calibration and quantitative element imaging**

253 Metal-spiked polymeric standards with theoretical concentrations of 0, 2.5, 10, 100 and 400
254 $\mu\text{g g}^{-1}$ (as major element) were scanned according to the conditions used for sample imaging.
255 For each standard, a set of 3 consecutive lines (ca 1.5-2 cm long) were scanned. Each ion
256 intensity was normalized by the respective internal standard intensity, and an average value
257 was calculated from the 3 scans for each standard. For each element, a linear regression was
258 obtained from these average values as a function of the respective standard concentrations.
259 These linear regressions were used to convert raw data (element intensity to internal standard
260 intensity ratio) from sample analysis to elemental concentrations ($\mu\text{g g}^{-1}$) by taking into
261 account the difference in ablated volume (thickness of polymeric film and tissue thin-section)
262 as well as the densities of the polymeric film and tissue.

263

264 **Data processing**

265 An imaging experiment for a single sample consisted of n csv files, which were then
266 concatenated. Then, a homemade program under Python was used to generate an elemental
267 spectrum and images of element concentrations per pixel using a color code (png image
268 format).

269

270 **Results and discussion**

271 The method relies on the use of polymeric films doped with metallic (including internal
272 standards) solutions for calibrants or internal standards for samples. Therefore, elements
273 present in biological tissues deposited on top of the polymeric film are quantified by internal
274 calibration after complete ablation. For reliable quantification, calibrants must be physically
275 and chemically characterized.

276 **Film characterization**

277 The main idea was to develop a method using a hydrosoluble polymer to extend the
278 methodology to a larger panel of metals likely to be encountered in living organisms but also
279 to avoid the use of organic solvents and thus reduce chemical waste treatment for a more
280 environmentally friendly and durable methodology. Furthermore, successful quantification
281 using polymer film calibrant strictly relies on two keys parameters: the chemical purity in
282 terms of metals of the polymer molecule and the homogeneity of the film produced.

283 Chitosan is a natural polysaccharide produced from chitin of skeletons of crustaceans and
284 cephalopods and the cell wall of mushrooms. It is soluble in slightly acidified water and
285 therefore was considered as a potential candidate in a first attempt to produce the polymeric
286 film. However, chemical analysis of a homemade chitosan compound (M_w 20 000 $g\ mol^{-1}$)
287 using ICP MS revealed high concentrations of Fe (ca 300 000 $\mu g\ kg^{-1}$), Ni (ca 3 000 $\mu g\ kg^{-1}$), Zn
288 ($750\ \mu g\ kg^{-1}$), Cu ($400\ \mu g\ kg^{-1}$), Mo ($100\ \mu g\ kg^{-1}$) and Pt ($1\ \mu g\ kg^{-1}$), preventing its use for the
289 purpose of this study as it would increase the chemical background during analysis. A set of 6
290 commercially available dextran polysaccharides that were 100% water soluble and with M_w
291 ranging from 50 000 to 500 000 $g\ mol^{-1}$ were then investigated. The determination of their
292 chemical composition by ICP MS allowed the selection of one dextran molecule (M_w 500 000
293 $g\ mol^{-1}$) with the lowest concentrations of elements of interest (P $\sim 73\ 000\ \mu g\ kg^{-1}$, Fe ~ 2800
294 $\mu g\ kg^{-1}$; Cu $\sim 180\ \mu g\ kg^{-1}$; Ni $\sim 148\ \mu g\ kg^{-1}$; Zn $\sim 77\ \mu g\ kg^{-1}$; Mo $\sim 51\ \mu g\ kg^{-1}$; Se $\sim 21\ \mu g\ kg^{-1}$; Co
295 $\sim 8\ \mu g\ kg^{-1}$; Cd $\sim 0.2\ \mu g\ kg^{-1}$; Hg $\sim 0.2\ \mu g\ kg^{-1}$ and Pt < LOD of ca. $0.02\ \mu g\ kg^{-1}$).

296 Polymer concentration and spin-coating parameters (speed and acceleration speed, rpm;
297 duration, s; N_2 gas stream), which impact film thickness and roughness, were optimized to first
298 obtain a visible homogenized film. Although these dextran films are transparent, defaults were
299 apparent when the wrong spin-coating conditions were used. Of note is the importance of the
300 cleanness and purity of the glass slide (dust deposits must be meticulously removed, and
301 chemical content must be assessed). Once default-free polymer films were obtained (i.e., at
302 $60\ mg\ mL^{-1}$; 4000 or 4500 rpm for 1 s), the homogeneity of the polymer film was assessed
303 using LA-ICP MS by scanning in imaging mode a large surface of several polymer substrates
304 ($n=2$ @ 4000 rpm and $n=2$ @ 4500 rpm) used for sample deposits and monitoring internal
305 standard (In, Rh, and Ir) signals. Over the 4 film substrates analyzed, internal standards signal
306 stability was in the range of 8 to 16% and was slightly lower for film substrates produced at
307 4000 rpm (RSD $\sim 12\%$) compared with film obtained at 4500 rpm (RSD $\sim 9\%$). For the latter
308 condition, RSD between the 2 film substrates was better for In (10%) compared with Ir (12%)
309 and Rh (14%). Therefore, spin-coating at 4500 rpm provided homogenized film substrates.

310 Elemental concentrations in film substrate were determined after the spin-coating step, which
311 involves evaporation of the solution, leading to a metal preconcentration. For film calibrants
312 at 0, 2.5, 10, 50, 200 and $400\ \mu g\ g^{-1}$ (expressed for major elements), concentrations
313 determined by total analysis of the solubilized film are presented in **Table 1** for all elements
314 present in polymer films taking into account the measured mass of each film. Compared to

315 the theoretical concentration expected in the film, preconcentration factors (PF) for each
316 element were determined and varied between 1 and 53 (average value by element)
317 depending on the element. The low Hg concentration suggested a loss of Hg during the
318 evaporation process such that no loss during filtration was observed. In addition to Hg, P and
319 Fe exhibited the highest variabilities (respectively 74 and 112% RSD) in terms of
320 preconcentration factors most likely due to measurement bias in the blank and at low
321 concentrations, reflecting contamination issues (i.e., PF= 63 and 158 for P and Fe, respectively,
322 at 2.5 $\mu\text{g g}^{-1}$). For the others elements, i.e., Ni, Co, Cu, Zn, Se, Mo, Cd, Pt and the three internal
323 standards (In, Rh, and Ir), preconcentration factors were in the range of 12 to 22 with RSD
324 between 4% (Pt) and 34% (Ni). These preconcentration factors are in the same order of
325 magnitude as that reported by Austin et al [25], who determined a 10-fold increase for Cu and
326 Zn. The mass of the film varied between 99 and 126 μg from the blank to the 400- $\mu\text{g g}^{-1}$
327 standard. This finding was consistent with the thickness of 40 nm (variance 1%) and 80 nm
328 measured for the blank and highest concentrated calibrant, respectively. This thickness
329 difference is potentially due to a slight acid content difference (from the solution of elemental
330 standards) between the blank and 400 $\mu\text{g g}^{-1}$ standard. This thickness determined approaches
331 values (9 ± 1 nm) obtained by means of the sputtering method [23, 29]. Thinner film is more
332 preferable compared with that previously reported [24] as surface variation would be
333 insignificant given the complete ablation of the film and μm spatial resolution of the laser.
334 Thinner films would also minimize the contribution of the chemical composition of the
335 polymer to the blank background. Therefore, quantification was performed based on
336 preconcentration factors determined for each element and the 40-nm thickness (density was
337 therefore determined to 3.6982) of the film, which is the substrate used for sample
338 deposition.

339

340 **Calibration and normalization**

341 The purpose of this study was to identify the most appropriate internal standard (supposedly
342 absent from biological samples) for each element taking into account its mass and FIP (eV).
343 Considering these two latter parameters, ^{115}In (5.79 eV), ^{103}Rh (7.46 eV), and ^{192}Ir (8.97 eV)
344 were investigated as internal standards as they cover the large range of mass and FIP of the
345 elements of interest. The responses of all elements under study were plotted as a function of
346 their concentration and the internal standard considered (**Figure 1**). Good correlation

347 coefficients (0.995 - 1) were obtained except for P (<0.5), Fe (<0.2) and Hg (<0.09). For Hg, as
348 expected from preconcentration factor determination, this lack of linearity originates from Hg
349 loss during spin-coating. Several attempts to stabilize Hg in solution using an excess of chloride
350 ions or cysteine ligand have been made but were not successful and require further research.
351 The poor correlation coefficients of P and Fe calibration result from the presence of these
352 elements in the polymer as it is observed in the blank and the lowest concentrations (2.5 and
353 10 $\mu\text{g g}^{-1}$). Furthermore, the presence of P and Fe in the glass slide (which is also ablated at
354 the surface (<10 μm depth)) might also contribute to increase the background, thereby
355 degrading the linear correlation at low concentrations. For these reasons, these three
356 elements have been excluded from the following discussion.

357 Regardless of the element, calibration curves show that In is the internal standard with the
358 highest sensitivity when normalization was applied. Compared to In calibration, reductions of
359 approximately 1.5 and 3.4 were observed when element responses were normalized with Rh
360 and Ir, respectively, and regardless of the element. This finding prompted us to first conclude
361 that In is the best internal standard for highest sensitivity, for all elements and independently
362 of the mass and FIP of the elements to be quantified. This feature is most likely due to its lower
363 FIP leading to a better response than Rh and Ir in ICP MS. At constant sensitivity parameters
364 (mainly ICP MS cones status), the graphs revealed good repeatability ($\text{SD} < 1.1$) for all element
365 concentrations and regardless of the internal standard for the same calibration analyzed thrice
366 weekly. Our data also demonstrated the stability and linearity of a calibration over one year,
367 ensuring the integrity of the polymeric film.

368

369 **Influence of ICP conditions**

370 ICP conditions were optimized for robust plasma and in an attempt to reduce potential
371 differential elemental responses due to ablation of different materials (film, tissue
372 (heterogeneous itself)), which might lead to incorrect quantification due to the introduction
373 of organic material into the plasma (carbon effect). First, adjustment of ICP power revealed
374 that the analysis could be run at 1400 until 1500 W, which favor sensitivity and signal stability
375 (supporting text, Figures 2-SI and 3-SI).

376 The addition of O_2 into the ICP was investigated to reduce carbon deposits and possible matrix
377 effects. First, the impact of O_2 addition was measured on calibration standards for all elements
378 (**Figure 2a and Figure 4-SI**). When O_2 is added, three groups of elements could be

379 differentiated according to their responses and the internal standard used for normalization,
380 i.e., (1) a group of elements benefiting from sensitivity improvement regardless of the internal
381 standard used; (2) a group of mixed elements with increased/stable or stable/decreased
382 sensitivity as a function of the internal standard used; and (3) a group of elements suffering
383 from decreased sensitivity with all internal standards. In the first group, O₂ addition enhanced
384 the sensitivity of Co and Mo normalized with In, Rh or Ir. From the second group, the sensitivity
385 of Ni and Cu increased when normalized with In and Ir, whereas sensitivity tends to decrease
386 with Rh normalization. From the same group, the sensitivity of Pt is maintained with Ir as
387 internal standard but tends to decrease when Pt is normalized with In and Rh. In the last group,
388 the sensitivities of Zn, Se and Cd decrease systematically with all internal standards. The
389 tendency of these 3 groups is likely to follow the FIP of these elements with the lowest FIP for
390 Co (7.88 eV) and Mo (7.09 eV) followed by Ni (7.64 eV)-Cu (7.73 eV) and Pt (8.96 eV) at the
391 limit of the 3rd group and finally the triplet of Zn (9.39 eV)-Se (9.75 eV)-Cd (8.99 eV). Therefore,
392 if O₂ is added, Ir offering close mass and FIP similar to those of Pt and should be considered as
393 the most appropriate to maintain analytical performances and reduce possible matrix effect.
394 Definitely, the use of O₂ in the ICP source must be avoided for the LA-ICP MS analysis of Zn,
395 Se and Cd unless more convenient internal standards (with closer mass and FIP) can be
396 identified that are not naturally occurring in biological samples.

397 The effect of O₂ addition for potential matrix effect reduction was studied during ablation of
398 biological tissue (homogenized metal-spiked kidney), which is a source of carbon introduced
399 in the ICP. **Figure 2b and 2c** shows elemental images of kidney thin-sections without the
400 addition of O₂ into the plasma (upper half part of the image) and with the addition of O₂ (lower
401 half part). A representative image illustrates the tendency for each group of elements as
402 defined above without internal standard normalization (2b) and with normalization (2c). For
403 the first group of elements, O₂ addition enhanced Co signal intensity even in the presence of
404 organic material. However, when Rh normalization was applied, no difference in relative
405 responses were observed between the two conditions of plasma, indicating that these
406 conditions were free of matrix effects. From the second group of elements, Pt exhibited
407 increased signal intensity with O₂ addition. In contrast, In normalization tended to decrease
408 the discrepancy of the Pt relative response over the thin-section regardless of plasma
409 conditions, although Pt/In responses were still slightly more pronounced at the upper part of
410 the section. With Ir normalization of Pt signal, no differential response could be observed

411 between the two statuses of O₂ plasma. Zn images that represent the thin section of the last
412 group demonstrated strong loss of sensitivity under O₂ plasma that internal standard
413 normalization did not recover, but Ir reduced the most, the differential response between the
414 two plasma conditions.
415 Together with results of the O₂ effects on calibration curves, these data demonstrate that the
416 response of low FIP elements can be more easily corrected even in the presence of organic
417 material and O₂ in the plasma, whereas high FIP elements are dramatically affected by the
418 presence of O₂ and carbon load in the plasma that cannot be compensated for by
419 normalization with any internal standard considered in this study. The intermediate group of
420 elements (Cu, Ni and Pt) requires careful attention in the selection of the most suitable
421 internal standard based on the plasma condition.

422

423 **Influence of laser energy and tissue thickness**

424 Thin sections of homogenized metal-spiked kidney (the matrix matched standard) were used
425 to assess the effect of laser energy delivered and tissue thickness (10 and 30 μm) on elements
426 response with and without O₂ in the ICP plasma. In most bioimaging studies, laser energy
427 ranges in the low J cm⁻². The two levels of energy (3.3 and 5.5 J cm⁻²) used in this study were
428 chosen to enable complete ablation of the tissue and limit glass slide surface ablation as much
429 as possible (**Figure 3**). On a 10-μm section analysis, these two levels of energy did not reveal
430 notable differences for Co, Pt and Zn relative responses, which are representative elements
431 of the three previously defined groups. On the other hand, when O₂ was added to the plasma,
432 all relative responses of Pt and Zn were decreased by at least 2-fold with a 1.6-fold energy
433 increase, whereas relative responses of Co were much less affected by O₂ while increasing
434 energy level. Therefore, given that increasing the laser energy did not favor sensitivity
435 enhancement and that a white deposit appeared on the skimmer cone after several days of
436 imaging runs, the lowest laser energy was chosen to reduce (in depth) glass ablation and thus
437 minimize white deposits originating from glass silicon oxide.

438 The relative response of the elements was compared for the two thicknesses of the matrix-
439 matched standard using 3.3 J cm⁻² fluency. In the first group, Co relative responses increased
440 proportionally (2.6 to 3-fold) with increases in section thickness for the majority of conditions
441 (In, Rh, Ir, +/- O₂). Mo behaved similarly to Co with or without addition O₂ to plasma.
442 Considering Pt from the 2nd group, the lowest correlation (3.7-fold proportionality) was

443 obtained when the Pt signal was normalized by Rh in the absence of the addition of O₂ to
444 plasma (2.9-fold for In and Ir normalizations). Under O₂, the proportionality between tissue
445 thickness increases and Pt normalized responses was slightly greater than with 3.6- to 4-fold
446 relative response increases (as noted for Cu and Ni). Concerning the 3rd group, the normalized
447 responses of Zn were on average poorly proportional (min-max: 1.7- to 2.9-fold) to the
448 increase in tissue thickness. The closest values (2.87 and 2.95-fold) were obtained under Rh
449 normalization without and with O₂ as observed for Se and Cd. Therefore, for this group of
450 elements, thinner sections (i.e., 10 μm) are preferred for LA-ICP MS analysis without the
451 addition of O₂ to plasma.

452 In summary, for the majority of these elements, with the exception of those in the third group
453 (Zn, Se, and Cd), the carbon load from biological tissue does not hamper LA-ICP MS analysis
454 on 30-μm sections given correct selection of internal standard for signal normalization.
455 Overall, this finding underlines the consistent behavior of elements within each defined group,
456 revealing once more that FIP is a key parameter to cope with the differences in carbon load in
457 the plasma and regardless of its composition (-/+ O₂).

458

459 **LA-ICP MS methodology validation**

460 The reliability of the developed methodology was assessed using the matrix-matched
461 standard, and homogenized kidney spiked with different amounts of metals (i.e., 2.5 μg g⁻¹,
462 50 μg g⁻¹ and 250 μg g⁻¹) was cryosectioned at 8, 10, 14 and 30 μm (n=3/thickness conditions
463 deposited in 3 different films). For each condition, 5 scans were acquired per kidney thin
464 sections. In parallel, metal concentrations in each kidney standard (n= 3/conditions) were
465 determined by conventional ICP MS analysis both in fresh and dried standards. Limits of
466 detection of the LA-ICP MS methodology (under laser sampling conditions used) were
467 determined according to the linear regression obtained by means of the polymer film
468 standards (i.e., LODs = 3SD of the blanks/slope) and by taking into account the densities of the
469 polymer film (d film = 3.6982 and d tissues=1), the thickness of the film (40 nm) and tissue
470 section (8-30 μm). Using In as an internal standard for an 8-μm thin section samples (the
471 thinnest section experimented), the limits of detections were in the μg g⁻¹ range and lower
472 with Co (0.1 μg g⁻¹), Mo (0.1 μg g⁻¹), Pt (0.1 μg g⁻¹), Cu (0.2 μg g⁻¹), Cd (0.3 μg g⁻¹), Ni (0.5 μg g⁻¹),
473 Zn (0.9 μg g⁻¹), and Se (1 μg g⁻¹). Accordingly, LOQs were approximately 3.3-fold increased.
474 These values are in the expected range as LODs are usually reported at the 10⁻⁴ to 10⁻¹ μg g⁻¹

475 level [16]. LODs of some hundreds of ng g^{-1} and $0.08 \mu\text{g g}^{-1}$ were reported for Cu [23] and Pt
476 [19], respectively, and are close to our values. However, LODs are not reported for Co, Mo,
477 Cd, Ni, Zn and Se for LA-ICP MS bioimaging analysis.

478 **Figure 4** presents LA-ICP MS quantification data obtained by means of metallic film calibration
479 using In as internal standard, 3.3 J cm^{-2} fluency and no O_2 in the ICP plasma. The LA-ICP MS
480 data are bracketed by concentration values in fresh and dry weight samples for each
481 condition. First, for all elements and thickness sections, concentrations determined by LA-ICP
482 MS are located between the values expressed as fresh weight (FW) and as dry weight (DW).
483 Of note, higher concentrations were obtained for 8- μm sections compared with 10- μm and
484 30- μm sections. This general trend is observed for all elements and is particularly pronounced
485 for the highest concentration of kidney standard where an average 2.8-fold decrease in
486 concentration is observed between the 8- and 30- μm sections. For the two other kidney
487 standards, this effect is reduced for all elements with an average 1.7- and 1.2-fold decrease at
488 2.5 and $50 \mu\text{g g}^{-1}$, respectively. For all kidney standard concentrations, Pt is the element least
489 affected by this decrease with 22% RSD (as Cd), 2% RSD (as Mo), and 64% RSD (as Cd, Mo and
490 Se) at $2.5 \mu\text{g g}^{-1}$, $50 \mu\text{g g}^{-1}$ and $250 \mu\text{g g}^{-1}$, respectively. In contrast, Ni is the most affected with
491 the highest concentration discrepancies between the 8- μm and 30- μm sections (69% RSD at
492 $2.5 \mu\text{g g}^{-1}$; 71% RSD at $250 \mu\text{g g}^{-1}$). However, these decreases does not seem to be mass
493 element dependent.

494 Additionally, at $250 \mu\text{g g}^{-1}$, quantification values obtained for an 8- μm sections are closer to
495 the DW concentration values than the FW values, especially for Co, Ni, Se and Mo with an
496 approximately 37% bias. While Cu and Zn concentrations are approximately one-half of the
497 value of DW, Zn and Cd values are one-half of an order of magnitude below the DW values.
498 Consequently, for this concentration standard, the bias of 30- μm section values with the FW
499 values are in the range of 5% (Pt) to 29% (Co) for most elements with an average bias of 11%
500 without taking into account Se (31%), Cd (49%) and Zn (56%). For the $50 \mu\text{g g}^{-1}$ kidney standard,
501 the average bias for the Co, Ni, Cu, Mo and Pt concentration values determined is 7%, and bias
502 for Zn, Se and Cd is increased (42-92%). At $2.5 \mu\text{g g}^{-1}$, the bias of the 30- μm section
503 concentrations to the FW concentration values increases (>30%), which could be explained by
504 the fact that the concentrations determined are moving closer to the LOQs. Nevertheless, the
505 Pt concentration is still the closest to the FW value (29% bias). Using Ru and Y as internal
506 standards for normalization in 20- μm sections and comparing concentrations determined by

507 LA-ICP MS and ICP MS, a slightly higher bias (35%) was obtained for Cu (approximately the
508 same level as $2.5 \mu\text{g g}^{-1}$), but the bias tended to be much lower for Zn (11% RSD at a
509 concentration of $8 \mu\text{g g}^{-1}$) [24], in contrast to our study.

510 To summarize, Pt is the least problematic element in contrast to Zn (followed by Cd), which
511 exhibits completely different behavior and appears to be difficult to properly quantify.
512 Given that the kidney sample is directly analyzed without a drying step, one could expect a
513 value closer to that expressed as fresh weight. This notion is likely to reflect the drying of the
514 biological sample due to high He flow in the ablation cell during the imaging run or during the
515 storage of the section before analysis. Thus, thinner sections would dry faster than the thicker
516 during the ablation process. The use of a cryocell would be beneficial to better control this
517 parameter in fresh biological samples analysis. Another alternative would be to dry (under
518 light vacuum for example) the biological section immediately after its deposition on the
519 polymer film without damaging the film substrate. In summary, the values determined by LA-
520 ICP MS fall within intervals of fresh-dry weight concentrations, ensuring the validity of our
521 methodology. In addition, the bias with the FW value can be assessed according to the
522 thickness of the section under such analytical conditions.

523

524 **Application to human clinical samples (ovaries and tumoral tissues)**

525 The methodology was applied to map elements in two types of samples from different
526 patients. The standard protocol for woman treated by Hyperthermic Intraoperative Peritoneal
527 Chemotherapy (HIPEC) for a peritoneal carcinomatosis is the removal of both ovaries. The
528 HIPEC protocol involves the intraperitoneal application of a circulating bath of a
529 chemotherapeutic solution heated at 42°C for 30 minutes. Platinum salts (cisplatin or
530 oxaliplatin) are the onco-drugs most commonly used in this protocol. We have access to
531 peritoneal tumoral nodules and ovaries resected before and after HIPEC. **Figure 5** shows the
532 quantitative distribution of Pt derived from the drug, endogenous elements (Cu, Zn and Mo)
533 and Cd. Of note, the qualitative and quantitative distributions were identical for all these
534 elements, confirming that polymeric film and internal standards were homogeneous even
535 after the drying step under a light vacuum. The highest concentrations ($15\text{-}20 \mu\text{g g}^{-1}$) of Pt
536 were mostly determined at the periphery of the ovary given the type of treatment, which
537 facilitated direct contact of the drug with the tissues and organs. In addition, approximately
538 $5\text{-}10 \mu\text{g g}^{-1}$ of Pt spots could be quantified in some central parts of the ovary, which potentially

539 correspond to residual Pt from prior systemic chemotherapy. Zn is homogeneously distributed
540 in the ovary at approximately $10 \mu\text{g g}^{-1}$. However, considering the quite important bias
541 determined from the kidney standard, this value is certainly underestimated. Cu is also
542 homogeneously distributed at lower concentrations, i.e., $2.5\text{-}5 \mu\text{g g}^{-1}$. Mo, an element involved
543 in some biological processes, was also detected at quantifiable ($0.1 \mu\text{g g}^{-1}$) levels but close to
544 background level. The presence of $1\text{-}2 \mu\text{g g}^{-1}$ Cd, which is quite unexpected, leaves us with
545 unanswered questions about its origin and its potential toxic effects on ovary functionality
546 [30].

547 This methodology was also applied to paraffin-embedded biological tissues, which is the most
548 common protocol used for the preparation of tissue sections for the creation of clinical tissue
549 banks. As shown in **Figure 6**, this preparation involving paraffin and polymer film immersion
550 in a warm bath did not hamper quantification given that internal standards remained
551 homogeneous after this entire process. Quantitative maps could be obtained for Pt, Cu, Zn
552 and Ni among all the set of elements monitored. The others were under LODs. Although Pt
553 was primarily found in the tissues surrounding the ovary, Pt concentrations were increased
554 and in the range of $30\text{-}60 \mu\text{g g}^{-1}$. In contrast to the ovary sample, the highest concentrations
555 ($30\text{-}90 \mu\text{g g}^{-1}$) of Cu and Zn were mostly noted at the periphery of the tumor tissue where
556 some ($10 \mu\text{g g}^{-1}$) Ni was also quantified.
557 This bioimaging analysis allowed to assess the penetration of Pt drug into tissues of patients,
558 thus enabling comparisons of clinical protocols and their efficacy in a long term study. Finally,
559 the quantification of endogenous elements (Cu, Zn, Ni, Mo) is also of paramount importance
560 as it could enable us to follow the potential impact of the drug on these essential elements.

561

562 **Conclusion**

563 Given that most bioimaging studies report quantitative mapping for one or two elements, the
564 developed methodology based on the use of a water-soluble polymer to produce metal-spiked
565 polymer film standards allows the simultaneous quantitative mapping of a set of eight
566 elements in biological samples for the first time. The 8 elements exhibited different behaviors
567 as a function of the internal standards used for normalization and experimental conditions for
568 analysis. Elements with low and medium first ionization potential ($<9 \text{ eV}$) can be easily
569 quantified in $8\text{-}30\text{-}\mu\text{m}$ tissue sections. For higher FIP elements, which are more subjected
570 to matrix effects, quantification is limited to $<10\text{-}\mu\text{m}$ sections. The methodology was validated

571 on kidney matrix standards, and LODs in the range of 0.1 - 1 $\mu\text{g g}^{-1}$ were determined for 8- μm
572 thin-sections. This quantitative bioimaging methodology was successfully applied to two
573 different samples following different sample preparations (fresh-frozen and fixed-embedded
574 sample) without impacting polymer film homogeneity, thus ensuring the use of this
575 methodology for clinical tissue banking.

576

577

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581

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688 **Table and figures caption**

689 **Table 1.** Chemical composition, weight and thickness of polymer film standards prepared by
690 spin-coating. Concentration ranges (0-400 $\mu\text{g g}^{-1}$ and 0-80 $\mu\text{g g}^{-1}$) are reported for major
691 elements (P, Fe, Cu, Zn, and Pt) and minor (Ni, Co, Se, Mo, Cd, and Hg) elements, respectively.
692 Rh, In and Ir are internal standards.

693 **Figure 1.** LA-ICP MS element calibration curves obtained as a function of internal standard
694 (Normalized Intensity on the Y-axis) after spin-coating and LA-ICP MS analysis.

695

696 **Figure 2.** Influence of O₂ addition to ICP plasma on **A)** Co, Pt and Zn sensitivity according to
697 internal standard (Y-axis: Normalized Intensity) and element responses normalized **C)** or not
698 **B)** during analysis of the 10- μm sections of matrix-matched kidney standard.

699 **Figure3.** Influence of laser energy and section thickness on element response according to the
700 three internal standard normalizations. Unless specified on the X-axis, experiments are run at
701 3.3 j cm⁻² fluency.

702 **Figure4.** Methodology validation with determination of element concentrations by LA-ICP MS
703 imaging in matrix-matched kidney standards at 2.5, 50 and 250 $\mu\text{g g}^{-1}$ (expressed as major
704 element concentrations) in 8-, 10-, 14- and 30- μm sections with In normalization.
705 Concentrations in fresh weight and dry weigh kidney standards were also determined by
706 ICPMS following acid mineralization of the standards.

707 **Figure 5.** Example of quantitative mapping of elements in an 8- μm section of a fresh frozen
708 ovary from patients under oxaliplatin treatment with a photography before LA-ICP MS
709 imaging..

710 **Figure 6.** Example of quantitative mapping of elements in an 8- μm section of a fixed and resin-
711 embedded tumor tissue from patients undergoing oxaliplatin treatment with a photography
712 before LA-ICP MS imaging.

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