

Probing the Ultrastructure of Spheroids and Their Uptake of Magnetic Nanoparticles by FIB–SEM

*Original*

Probing the Ultrastructure of Spheroids and Their Uptake of Magnetic Nanoparticles by FIB–SEM / Mollo, V., Scognamiglio, P., Marino, A., Ciofani, G., Santoro, F.. - In: ADVANCED MATERIALS TECHNOLOGIES. - ISSN 2365-709X. - STAMPA. - 5:3(2020), p. 1900687. [10.1002/admt.201900687]

*Availability:*

This version is available at: 11583/2801883 since: 2020-03-11T11:22:15Z

*Publisher:*

Wiley-Blackwell

*Published*

DOI:10.1002/admt.201900687

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1 **Probing the ultrastructure of spheroids and their uptake of**  
2 **magnetic nanoparticles by FIB-SEM**

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14  
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16 **MANUSCRIPT**  
17

## 18 **Abstract**

19 Spheroids are 3D cellular systems largely adopted as model for high-throughput screening of  
20 molecules and diagnostics tools. Furthermore, those cellular platforms also represent a model for  
21 testing new delivery carries for selective targeting. The coupling between the 3D cell environment  
22 and the nanovectors can be explored at the macroscale by optical microscopy. However, the  
23 nanomaterial-cell interplay finds major action at the single cell and extracellular matrix level with  
24 nanoscale interactions. Electron microscopy offers the resolution to investigate those interactions,  
25 however the specimen preparation finds major drawbacks in its operation time and preciseness. In  
26 this context, focused ion beam and scanning electron microscopy (FIB-SEM) allows for fast  
27 processing and high resolution of the cell-nanomaterial interface. Here, in fact, we show a novel  
28 approach to prepare large-area 3D spheroid cell culture specimens for FIB-SEM. We explored  
29 sectioning procedures to preserve the peculiar structure of spheroids and their interaction with  
30 magnetic nanovectors. Our results pave the way for advanced investigations of 3D cellular systems  
31 with nano and micromaterials relevant to tissue engineering, bioelectronics and diagnostics.

32

33 **Keywords:** scanning electron microscopy, focused ion beam, spheroids, biointerfaces,  
34 nanocarriers, endocytosis.

35

## 36 **Introduction**

37 In the last decade, spheroid-like cellular architectures have become powerful model systems to  
38 biomimic complex tissue-like forms towards the fully recapitulation of organoid-like systems. In  
39 fact, these closed-systems are excellent tools for understanding complex cellular functions, testing  
40 new molecules for drugs and diagnostics solutions<sup>[1]</sup>. In this context, 3D tumor spheroids have  
41 been adopted as reliable model of *in vivo* solid tumors for the screening of different anticancer  
42 drugs and nanoformulations<sup>[2,3]</sup>. In comparison to 2D cancer cell cultures, tumor spheroids display  
43 many different features of the *in vivo* solid tumors (*e.g.*, spatial architecture, high-level secretion  
44 of soluble mediators, gene expression profile and multidrug resistance mechanisms)<sup>[4]</sup>.

45 Our former studies, for instance, adopted spheroid-like tumoral system to evaluate the efficacy of  
46 lipid nanovectors have been loaded with superparamagnetic iron oxide nanoparticles (SPIONs)<sup>[5]</sup>.  
47 SPIONs efficiently generate heat when exposed to alternated magnetic fields (AMF) and have  
48 been successfully exploited for hyperthermia treatment of glioblastoma in clinical trials<sup>[6]</sup>.

49 Furthermore, our group has recently developed lipid nanovectors loaded with both SPIONs and  
50 the temozolomide (TMZ) drug for the combined hyperthermia and chemotherapy treatment of  
51 glioblastoma cells<sup>[7]</sup>. For all these aforementioned applications (i.e. hyperthermia, MRI imaging  
52 and chemotherapy treatment), the nanovector accumulation inside the 3D tumor models is of  
53 crucial importance.

54 In this scenario, these 3D closed- systems and their interaction with nanomaterials have been so  
55 far largely investigated by means of optical microscopy techniques<sup>[8,9]</sup>. However, there are  
56 certainly limitations in terms of characterization of the inner spheroid interactions, achieved  
57 resolution and labelling of multiple cellular components. Alternatively, 3D cell architectures can  
58 be further inspected at the nanoscale by electron microscopy techniques. In fact, the typical  
59 polymeric embedding ensures that the 3D morphology is preserved and the cross sectioning allows  
60 for the investigation of specific thin sections and eventually the reconstruction of a volume of  
61 interest<sup>[10]</sup>.

62 Transmission electron microscopy can be performed following a mechanical thin-sectioning,  
63 allowing for slice thickness in the range of  $\sim 70$  nm<sup>[11]</sup>. Given the large size of 3D spheroids, this  
64 procedure would yield to hundreds of sections which might be collected and analyzed individually.  
65 This is an extremely time consuming process and recently, scanning electron microscopy coupled  
66 with in-chamber mechanical serial sectioning tools have provided an alternative approach for fast  
67 processing and imaging <sup>[12]</sup>.

68 Here, serial block-face imaging scanning electron microscopy (SBF-SEM) and focus ion beam  
69 scanning electron microscopy (FIB-SEM) found major applications for morphological analysis of  
70 stem cell spheroids, organoids, as well as organotypic cell cultures.

71 SBF-SEM uses the automated ultramicrotome located inside the SEM chamber and removes  
72 sections ( $\geq 20$  nm thick) from the block face and provides scanning of relatively large volumes.

73 Alternatively, focused ion beam- scanning electron microscopy (FIB-SEM) tomography is the  
74 most promising approach for 3D imaging at the subcellular level and is considered as a revolution  
75 for ultrastructural volume reconstruction<sup>[13-15]</sup> avoiding the drawbacks of mechanical sectioning  
76 procedures and achieving sections' thickness  $< 20$  nm. In fact, this technique has been already  
77 adopted for the investigation of 2D and 3D cultures on diverse biomaterials<sup>[16-19]</sup> and the  
78 characterization of complex tissue-like architectures.<sup>[20,21]</sup>

79 In this work, we further exploit the FIB-SEM procedure to investigate regions of interest in 3D  
80 spheroid cell cultures and their interaction with magnetic nanovectors, which can penetrate and  
81 reach the spheroid inner domain. The spheroids have been macroscopically observed employing  
82 two specimens preparation procedures which include hard drying and resin-embedding processes.  
83 Given the large size of the spheroids, those have been initially mechanically sectioned to reduce  
84 their effective volume and be subsequently polished by focused ion beam. Finally, we characterize  
85 the interface between cell and extracellular matrix with magnetic nanovectors exploring the  
86 different phases of the SPIONs uptake process.

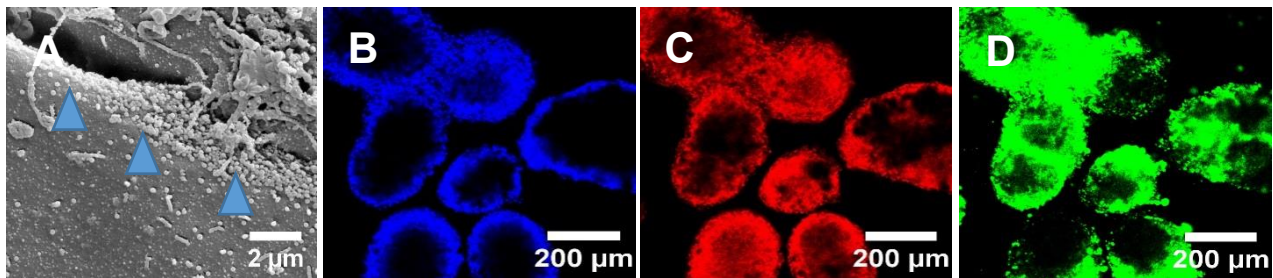
87

## 88 **Results**

89 3D spheroids have been obtained from U87 MG cells and assemble in a 3D architecture following  
90 the hanging-drop procedure (see **Experimental Section**). The U87 MG-derived spheroids were  
91 loaded with lipid magnetic nanovectors (LMNVs) labeled with a lipophilic fluorescent probe,  
92 which marked the lipid part of the vectors (24-48 hours of incubation). Nanovectors approach the  
93 surface of individual cells (**Figure 1A**, the scanning electron micrograph of spheroid also shows  
94 the interaction of LMNVs with its surface as shown by the blue arrows) and they further penetrate  
95 both in the intracellular and inter-cellular domains. In fact, **Figure 1B-D** show how cells and  
96 nanovectors simultaneously populate the spheroids.

97 Thus, through fluorescence imaging, the rate of penetration and the quantification of LMNVs  
98 inside the spheroids were calculated. In fact, after 24 hours 0.8 % (s.d.: +- 0.7 %) of the total  
99 volume of the spheroids were populated by LMNVs and after 48 hours this volume reaches 8.1%  
100 (s.d.:+- 0.5 %)<sup>[5]</sup>. To further evaluate the nanovector-spheroid interface interaction, we prepared  
101 the specimens for SEM/FIB milling and imaging.

102



103

104 **Figure 1: Morphology of U87\_MG derived spheroids and nanovector internalization: A)**

105 Scanning electron micrograph of spheroid surface treated with ROTO protocol with lipid magnetic

106 nanovectors, LMNVs (blue arrow). B,C,D) confocal laser scanning microscopy imaging of  
107 spheroids: nuclei in blue (Hoechst) with fluorescent nanovectors in green and F-actin in red; scan  
108 area is 1273  $\mu\text{m}$  x 1273  $\mu\text{m}$ .

109  
110 Due to the large size, sectioning *via* FIB of a whole spheroid (400-500  $\mu\text{m}$ ) would require  
111 extremely long processing times. For this reason, each spheroid was divided in to four parts during  
112 the fixation and embedding procedure. The cutting was carried out carefully with a small razor  
113 blade (see **Supplementary Information S1**). To assess the possible damages due to the  
114 mechanical sectioning (i.e. compression, breakages, eradication of organelles, etc.), the  
115 ultrastructure of the spheroids was investigated after each step of cutting.

116 The ROTO-UTP (reduced osmium–thiocarbohydrazide–osmium ultra thin plasticization) protocol  
117 used for the specimen preparation is structured in nine main steps (**Figure 2**, Box A), in relation  
118 to the use of different substances for fixation and heavy-metal staining (glutaraldehyde, glycine,  
119 osmium tetroxide/potassium ferro-cyanide, thiocarbohydrazide, osmium tetroxide, uranyl acetate,  
120 tannic acid, ethanol, Spurr’s resin). Among these procedure’s steps, six were selected as a point in  
121 time when each spheroid was sliced in to four parts. Thus, we identify six cutting steps: step 1: after  
122 fixation in glutaraldehyde; step 2: after osmium tetroxide; step 3: after thiocarbohydrazide (TCH);  
123 step 4: after tannic acid; step 5: in resin embedding before polymerization; step 6: in resin after  
124 polymerization as described in **Figure 2 (Box A-B)**. After each cutting step, the each spheroid  
125 section was embedded according the ROTO-UTP protocol. Here, spheroids appear comparably  
126 soft during the cutting after fixation in glutaraldehyde. In fact, samples’ hardness increased after  
127 the incubation with osmium tetroxide and thus the spheroids acquire resistance to cutting  
128 especially after the treatment with TCH (step 3).

129 It is known that the tannic acid used after glutaraldehyde and osmium tetroxide fixation improves  
130 the preservation of the cell features against shrinkage and thermal damage occurring during the  
131 sample preparation<sup>[22]</sup>. Here, when the tannic acid was added, the spheroids surface became fragile  
132 during the cutting (step 4) and at the outside part of the spheroid several cells collapsed (see  
133 **Supplementary Information S1**). After the embedding in Spurr’s resin, spheroids became more  
134 resistant by comparing them to the other spheroids sliced in previous steps and intact during the  
135 sectioning procedure (step 5), especially after the final polymerization (step 6). The size of the  
136 whole spheroid was not s appreciably affected during the ROTO-UTP protocol as shown in **Figure**

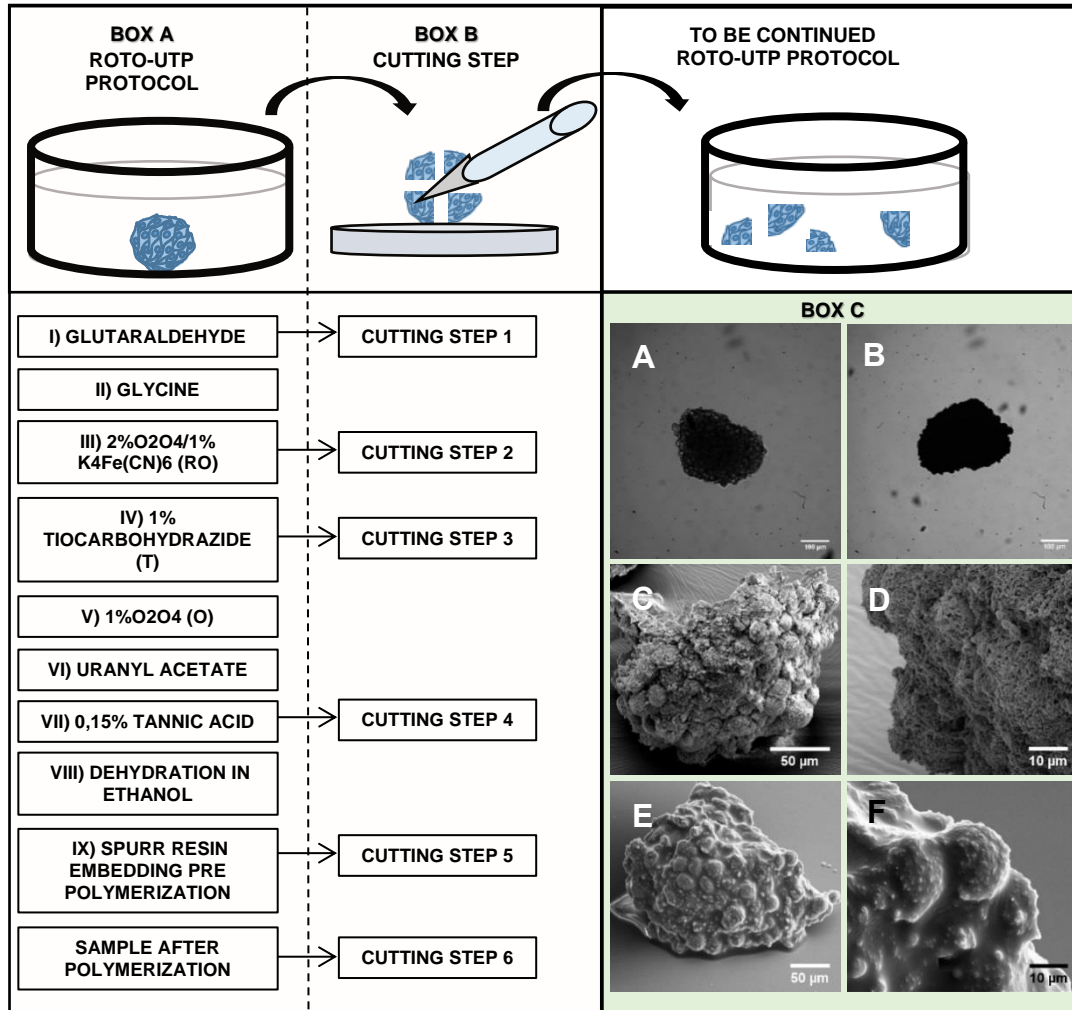
137 **2A** (post glutaraldehyde fixation) and **Figure 2B** (in Spurr's resin embedding) and further  
138 discussed in see **Supplementary Information S1**).

139 Complementarily, the dehydration procedure through critical point drying (CPD) was carried out  
140 on whole spheroids as shown in **Figure 2 C,D**. During the CPD process, the most crucial step is  
141 in the final part of the procedure where the outflow of the gas of the supercritical carbon dioxide  
142 is carried out slowly in order to avoid the collapse of samples. (0.05 bar/sec).

143 Effectively, U87-based spheroids exhibited a complex 3D structure which expands over several  
144 hundreds of micrometers. Furthermore, the overall morphology have been investigated by  
145 secondary electrons detection after the drying procedures. Interestingly, spheroids prepared *via*  
146 CPD had individual cell bodies which appeared round and homogenously distributed throughout  
147 the whole spheroid area with no comparable collapsed regions. Here, at higher magnification,  
148 apical microvilli protrusions are clearly visible (**Figure 2 C-D**), however, when FIB cross  
149 sectioning was performed (**Supplementary Information S2**) no intact cellular ultrastructures  
150 were distinguishable because of the cavities and artefacts induced by the hard drying procedure<sup>[23]</sup>.

151

152



153 **Figure 2: SCHEMATIC FLOW OF SPECIMEN PREPARATION.** Box A summarizes the  
 154 main points of the ROTO-UTP protocol (from I to IX) while in Box B the steps for the mechanical  
 155 sectioning of the spheroids are reported. To evaluate the U87-MG derived spheroids ultrastructure  
 156 a total of 18 spheroids were prepared with this procedure and, 3 spheroids were cut in four parts  
 157 by using a razor blade at each set time points. After cutting, each sliced spheroid underwent ROTO-  
 158 UTP procedure. Box C shows the size of an exemplary spheroid after fixation in glutaraldehyde  
 159 (A) and its final morphology in Spurr's resin by using the optical microscope (B) and scanning  
 160 electron microscope (E,F). C and D are scanning electron micrographs of whole U87-MG derived  
 161 spheroids acquired in secondary electrons mode after critical point drying.

162

163 In parallel, following the ROTO procedure<sup>[16]</sup>, a group of intact spheroids was embedded in Spurr's  
 164 resin. To reveal the structure of spheroids, the resin excess was removed from their surface by

165 washing with absolute ethanol for 3 seconds before the polymerization in oven at 70°C (**Figure**  
166 **2E,F**).

167 In this way, the resin penetrates the intracellular domain and allows for stabilization of the  
168 ultrastructures. In fact, the 3D architecture of the spheroids is preserved and the cells' cluster is  
169 clearly distinguishable. However, the final removal procedure might induce a nanometer thick  
170 layer of polymerized resin around the cellular bodies which covers features, i.e. microvilli, which  
171 are otherwise not visible in the spheroids prepared by CPD.

172 To reveal the inner architecture of the mechanically-sectioned spheroids, FIB milling was further  
173 performed on the resin-embedded specimens. First, a region of interest (ROI) was located and  
174 preserved by a platinum (Pt) layer deposited *via* ion beam. In some cases, depending on the  
175 geometry of the spheroid, a thick layer of gold (~ 50 nm) was deposited prior to the SEM  
176 observation and was appropriate to limit charging effects and preserve the ROI. This is in fact  
177 valid for smaller spheroids whose mechanical-cut subsections would have a final diameter of about  
178 100-200  $\mu\text{m}$ .

179 For larger subsections, first a thin film of platinum (~0.2  $\mu\text{m}$ ) was deposited with an electron  
180 current of 26 nA and a voltage of 30 kV covering a nominal rectangular area of 75  $\mu\text{m}$  by 40  $\mu\text{m}$   
181 (see **Experimental Procedure** and **Supplementary Information S3**). Subsequently, an ion-  
182 assisted Pt deposition was performed with an current of 9.3 nA and a voltage of 30 kV, in order to  
183 achieve a final Pt thickness of ~ 1 $\mu\text{m}$ . Moreover, thicker and more irregular spheroid subsections  
184 were covered with an additional Pt layer of ~ 0.2  $\mu\text{m}$ .

185 Then, a first large area trench out is performed to remove the material in the surrounding of the  
186 ROI.

187 A rectangular area of 75  $\mu\text{m}$  by 40  $\mu\text{m}$  was located, and the milling was performed with a current  
188 of 21 nA at 30 kV, fixing a nominal (for silicon) etching depth of 10  $\mu\text{m}$ . Depending on the  
189 structural composition of the spheroid subsections, the resulting cross section could be directly  
190 visualized by the backscattered electrons (BSE) detector, while an additional polishing step is  
191 carried out to reduce possible curtaining effect or material re-deposition<sup>[24]</sup>. We found that at least  
192 three subsequent polishing steps at decreasing currents and milling areas gave the best results in  
193 terms of smoothness and definition of the target cross section. In fact, we carried out serial-  
194 sequential milling steps by fixing currents at 0.79 nA, 21 nA, 93 nA, for areas of 75  $\mu\text{m}$  by 30  $\mu\text{m}$ ,

195 75  $\mu\text{m}$  by 20  $\mu\text{m}$ , 75  $\mu\text{m}$  by 10  $\mu\text{m}$ , respectively. The overall FIB cross section milling workflow  
196 is summarized in **Supplementary Information S3**.

197 The ROI visualization was finally achieved through the BSE detector and secondary electron  
198 modes (SE, see also **Supplementary Information S4**). However, BSE micrographs allow for high  
199 resolution ( $\sim 5$  nm, different acquisition currents/voltages have been tested as shown in  
200 **Supplementary Information S5**) and high contrast, enlightening areas with different material  
201 composition, density and conductivity within the spheroid.

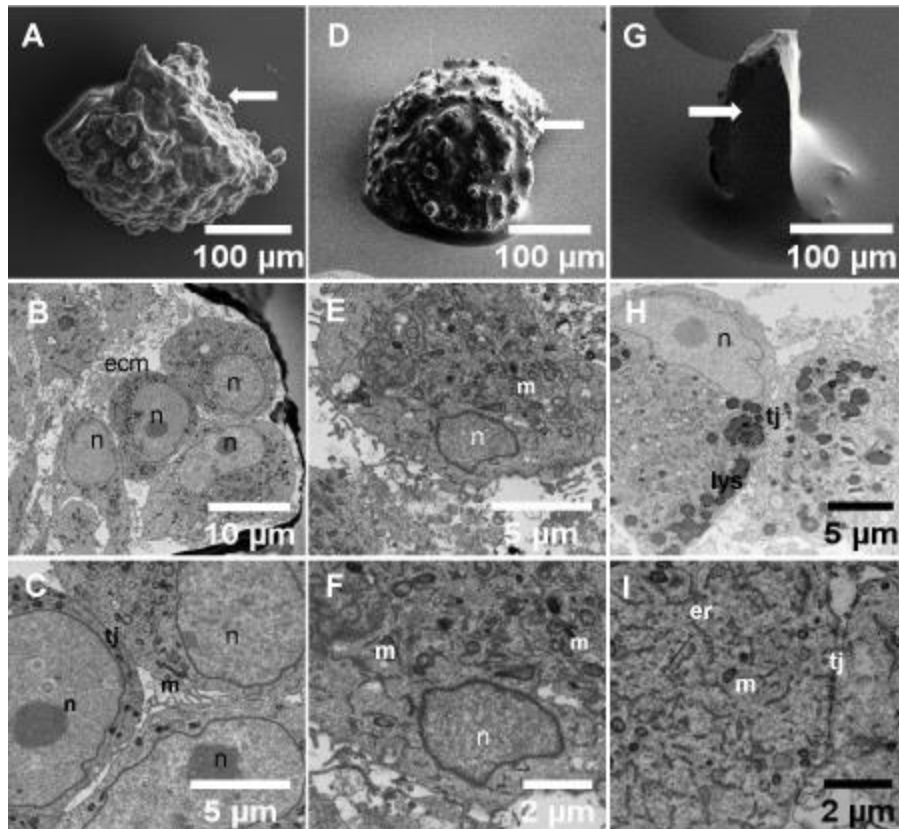
202 Here, by visualizing several cross sections, we can investigate the effect of the mechanical  
203 sectioning at different steps of the staining/embedding procedure. In **Figure 3** are reported the  
204 internal structure of spheroids after fixation in glutaraldehyde (step 1, **Figure 3A-C**), and in  
205 Spurr's resin embedding before (**Figure 3D-F**) and after polymerization (**Figure 3G-I**) which are  
206 named here as step 5 and step 6, respectively. SE micrographs in **Figure 3A,D,G** show an overview  
207 of a spheroid subsection where an exemplary cutting plane is identified (white arrows). Analogous  
208 micrographs have been also reported in **Supplementary Information S6-S7** and a set of  
209 sequential cross sections at a 20 nm pitch have been also collected to visualize a volume of interest  
210 (**Supplementary Movie 1**).

211 Furthermore, the BSE micrographs in **Figure 3** (see also **Supplementary Information S7**)  
212 revealed defined nuclei (N), large number of mitochondria (M) with appreciable crests, abundant  
213 rough endoplasmic reticulum (rER), extracellular matrix (EC) and tight junctions (TJ). No  
214 appreciable vacuoles or similar structures, which would indicate the eradication of organelles due  
215 to cutting were detectable<sup>[25]</sup>.

216 Comparing the inner and outer area of spheroids, we found evidence of compression effects due  
217 to mechanical stress as effect of the mechanical cutting when spheroids underwent only primary  
218 fixation (step 1).

219

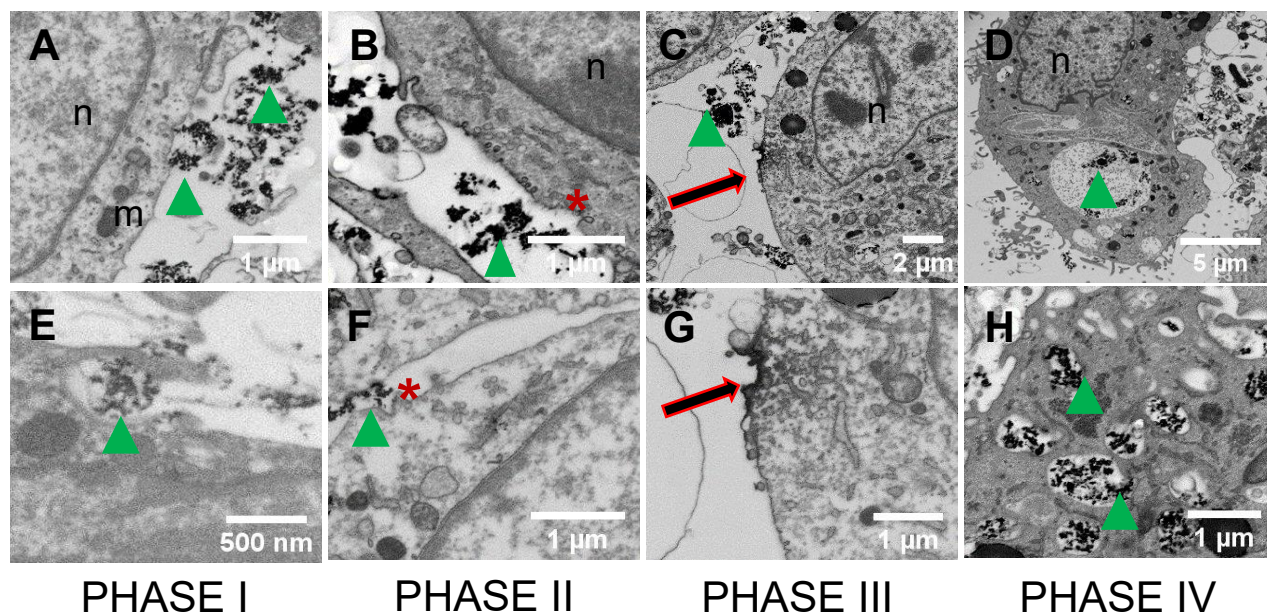
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221  
 222 **Figure 3: Characterization of spheroid subsections.** A-C) Scanning electron micrographs (BSE)  
 223 of U87-MG derived spheroids prepared with ROTO and UTP protocol and resulting cross sections  
 224 post glutaraldehyde cutting D-F) pre-polymerization cutting, G-I) post-polymerization cutting.

225 Spheroids subsections obtained after resin embedding prior to polymerization (step 6) were further  
 226 milled and polished as mentioned earlier.

227 Finally, we investigated the interface between LMNVs and the outer and inner domains of the  
 228 tumoral spheroids. The micrographs in **Figure 4E-H** display the different phases occurred during  
 229 the endocytosis of LMNVs. *Phase I* represents the first contact between the nanovectors and the  
 230 outer membrane (contact) followed by the formation of membrane invaginations (*phase II*) and  
 231 then the formation of specific (electron-dense<sup>[26]</sup>) clathrin-coated pits (*phase III*). **Figure 4H** shows  
 232 the final LMNVs internalization and their inclusion into vesicles (*phase IV*). In particular, high  
 233 magnification micrographs revealed the presence of nanovectors in the extracellular matrix  
 234 domain, in the cytoplasm and incorporated in vacuoles (see **Supplementary Information S8**).



236 **Figure 4: Investigation of nanovectors uptake in spheroids by SEM-FIB.** A-H) SEM  
 237 micrographs of U87-MG derived spheroids treated with ROTO-UTP protocol after incubation with  
 238 lipid magnetic nanovectors: backscattered electrons micrographs on inner spheroid cross sections  
 239 which exhibit diverse nanovectors uptake: phase I- contact; phase II: invagination; phase III:  
 240 clathrin; phase IV: internalization. N: nuclei, green arrow: LMNVs; black arrow: clathrin; V:  
 241 vacuoles, red star: invagination.

242

### 243 Conclusions

244 We showed a procedure for the investigation of 3D tumoral spheroids loaded with lipid magnetic  
 245 nanovectors. Here, the resolution of the interface allows for the characterization of the cell-  
 246 nanocarriers interaction and the different uptake phases taking place within the spheroid. In order  
 247 to proceed with localized milling and high resolution imaging, the 3D spheroids have been first  
 248 mechanically sectioned. The sectioning has been performed at different steps during the heavy-  
 249 metal staining and the embedding procedure and possible shear stress effects have been evaluated  
 250 by performing FIB-SEM milling and imaging. Furthermore, we could investigate the cell-cell and  
 251 cell-extracellular matrix interplay with nanometer resolution and, finally, evaluate the interaction  
 252 between 3D spheroids and lipid magnetic nanovectors. In conclusion, this study proves a fast  
 253 artefact-free processing for the nanoscale investigation of 3D complex cellular systems like

254 spheroids and organoids with nano and macromaterials which more and more are being developed  
255 for tissue engineering, bioelectronic and diagnostic platforms.

256

## 257 **Experimental Section**

258 **Spheroids culture.** Cancer spheroids were obtained from glioblastoma multiforme U87-MG cell  
259 line (ATCC® HTB-141TM). The composition of the cell medium was Dulbecco Modified Eagle's  
260 Medium (DMEM) supplemented with fetal bovine serum (FBS; 10%), L-glutamine (1%), sodium  
261 pyruvate (1%), non-essential amino acids (1%), penicillin (100 IU/ml), streptomycin (100 µg/ml).  
262 U87 MG cells were cultured in 75 cm<sup>2</sup> flasks with a 20-85 % confluence range. For obtaining the  
263 spheroids with the hanging drop method<sup>[27]</sup>, cells were treated with trypsin (0.05% for 5 minutes),  
264 centrifuged (300 RCF for 6 minutes) and then resuspended at 10<sup>6</sup> cells/ml concentration.  
265 Subsequently, 25 µl drops of cell suspension were deposited upside down on lids of 10 cm diameter  
266 Petri dishes and incubated at for 24 hours at 37°C, 5% CO<sub>2</sub> and 100% humidity. The obtained 2D  
267 cells aggregates were finally transferred to non-adherent supports consisting in 1% agarose-coated  
268 Petri dishes and cultured for 4 days with complete medium for obtaining 3D spheroids.

269 **Fluorescence imaging.** For fluorescence labelling and confocal fluorescence microscopy  
270 imaging, spheroids were transferred from Petri dishes to 24-well Ibidi®, washed three times with  
271 PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes at 4°C, labelled with TRITC-  
272 phalloidin (100 µM) and Hoechst 33342 (1 µg ml<sup>-1</sup>). Imaging was carried out with a confocal  
273 fluorescence microscopy (C2s system, Nikon) and 3D reconstruction of z-stacks was performed  
274 by using NIS Element software (Nikon).

275 **Nanoparticle loading.** Lipid magnetic nanovectors (LMNVs) were fabricated as previously  
276 described<sup>[7]</sup>, and spheroids were incubated for 24-48 hours with 167 µg/ml of LMNVs.

277 **Fixation and staining for EM.** The specimens were prepared following the ultra-thin  
278 plasticization (UTP) procedure previously described<sup>[17,28]</sup>. Spheroids were washed once with 0.1  
279 M sodium cacodylate buffer (EMS, pH 7.2) and then fixed in 2.5% glutaraldehyde solution  
280 (Electron Microscopy Sciences, EMS) in the same buffer overnight at 4°C. After washing three  
281 times with 0.1 M sodium cacodylate buffer, sample were post fixed in 2% osmium tetroxide/1%  
282 potassium ferrocyanide (EMS, RO step), for 1 hour at 4°C in the dark and washed with buffer on  
283 ice. Then spheroids were washed with distilled water and kept in water until room temperature  
284 was reached. In the meantime, 1% thiocarbohydrazide solution (TCH, EMS) was prepared by

285 mixing the compound powder and distilled water and heated up at 60°C for 1 hour before filtration.  
286 Samples were incubated in TCH solution at room temperature for 20 minutes in the dark (T step)  
287 rinsed 3 times with distilled water and post-fixed in 1% osmium tetroxide (O step) for 1 hour at  
288 room temperature. After washing with distilled water, they were finally transferred in 0.5% uranyl  
289 acetate aqueous solution (EMS) overnight at 4°C in the dark. The next day, spheroids were washed  
290 in chilled water and incubated for 3 minutes in 0.15% tannic acid solution (Sigma Aldrich). After  
291 washing with water at 4°C, samples were dehydrated in ascending series of ethyl alcohol (Carlo  
292 Erba reagents, 30%-50%-70%-95%-100%). Each step was performed for 10 minutes at 4°C. 95%  
293 EtOH was performed two times while absolute ethanol step was performed two times at 4°C and  
294 for a last time 10 minutes at room temperature. For the sectioning of the spheroids, a razor blade  
295 with 30° micro knife (EMS) was used.

296 **Critical point drying.** After dehydration, samples were finally prepared for critical point drying  
297 (CPD) During the CPD sample preparation, spheroids were placed in a critical point chamber (EM  
298 CPD 300, Leica) keeping the level of ethanol to completely immerse the specimens. Then, ethanol  
299 was slowly exchanged with liquid CO<sub>2</sub> at 15 °C and 25 cycles of fluid exchange were performed.  
300 The heating up process to generate supercritical CO<sub>2</sub> was carried out at 37°C. Starting from 31°C,  
301 the supercritical CO<sub>2</sub> turns in to gaseous CO<sub>2</sub>. The gas CO<sub>2</sub> is then let out of the chamber through  
302 a dedicated valve.

303 **Resin embedding.** Spheroids were embedded in epoxy resin (ER) according to the ultra-thin  
304 plasticization protocol<sup>[16,29]</sup> after the dehydration step. The embedding was carried out by  
305 exchanging 100% ethanol solution with a mixture of absolute ethanol/Spurr's resin (EMS) with  
306 the following ratios and duration: 3:1 for 2 hours; 2:1 for 2 hours; 1:1 overnight; 1:2 for 2 hours;  
307 1:3 for 2 hours. Finally, the mixture was replaced with absolute resin in which spheroids were  
308 incubated for one night. Sample were finally embedded in fresh Spurr's two times (3 hours per  
309 each step) before polymerization. To avoid damages to the spheroid structure, a glass capillary  
310 with a sealed tip was used to collect them from resin and move them to round glass coverslip. Each  
311 coverslip was kept in vertical position for 2-3 hours to allow for the excess resin to drain. After  
312 the polymerization at 70°C for 24 hours, samples were mounted on a 12 mm aluminum stub using  
313 conductive silver paste (RS Company).

314 **SEM/FIB.** Samples were covered with a 10-20 nm-thick gold layer *via* sputter coating.  
315 Afterwards, they were loaded into a dual beam machine (Helios 650, Thermo Fisher Scientific).

316 The spheroid surface was scanned with the electron beam at a voltage of 3-5 kV (secondary  
317 electrons) to identify a region of interest (ROI).

318 Once a ROI was located, a first 0.2  $\mu\text{m}$ -thick platinum layer was deposited *via* electron beam-  
319 assisted deposition by setting a voltage at 3kV and a current in the range 0.79-9.3 nA. Afterwards,  
320 the sample was tilted at 52° to be perpendicular to the ion beam and a second layer of Pt was  
321 deposited by ion beam-assisted deposition to reach a final thickness of ~1  $\mu\text{m}$  (in some cases a  
322 deposition of an additional 200 nm thick Pt layer was necessary) .

323 A rectangular-shaped area was located for the milling. Here, the length was always kept at 75  $\mu\text{m}$   
324 while the width varied (depending on the milling step, see **Supplementary Information S4**) in  
325 the range 40 – 10  $\mu\text{m}$  and the etching depth was nominally (as for silicon) 10  $\mu\text{m}$ . The ion milling  
326 was carried out fixing a voltage at 30 kV and a current in the range of 65 nA-80 pA.

327 Furthermore, possible curtaining effects and material re-deposition were compensated through  
328 milling with lower currents (from 80 pA to 0.23 nA).

329 Image acquisition was performed in backscattered electrons mode fixing the dwell time at 30  $\mu\text{s}$ ,  
330 2 kV as voltage and 0.23 – 0.69 nA as current (dynamic focus built-in function turned on).

331

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- 385

## 386 **Acknowledgements**

387 The authors thank Laura Matino for the help with the UTP procedure and the staff of the  
388 Cleanroom Facility at the Central Lab of Istituto Italiano di Tecnologia for the use of the dual-  
389 beam microscope.

390

391

392 **Probing the ultrastructure of spheroids and their uptake of**  
393 **magnetic nanoparticles by FIB-SEM**

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395

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405 **SUPPLEMENTARY INFORMATION**

406

407

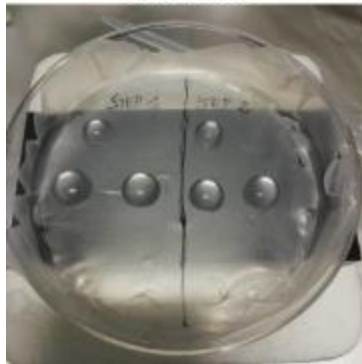
408 **Supplementary Movie 1:** sequential cross sectioning of a resin embedded spheroid (mechanical  
409 cutting performed after resin infiltration) was carried out. 50 frames with a 20 nm pitch were  
410 collected. The video is assembled with a speed of 4 frames/sec.

411

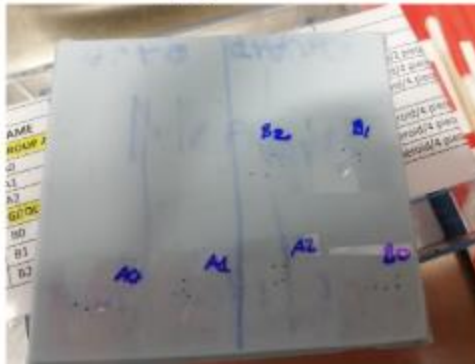
#### 412 **S1 -Technical information concerning cutting steps of spheroids.**

413 Spheroids were divided in to four parts using a razor blade with an orientation of 30°. The  
414 procedure was carried out under chemical hood with a stereo-microscope. For the cutting,  
415 spheroids were put on a black support on ice to optimize their visualization and keep them  
416 refrigerated at the same time. After that, all the ROTO and dehydration steps were carried out in  
417 drops laid on Parafilm and the pieces of spheroids were picked up in a minimum volume (2-5 µl).  
418 The cutting carried out after the primary fixation (step 1) is challenging because the spheroids are  
419 soft and quite transparent. After staining with osmium tetroxide and potassium ferrocyanide both  
420 handling and visualization became easier, thanks to the contrast conferred by osmium and at the  
421 same time the chemical substance makes the cellular structure more compact and stable, especially  
422 the plasma membrane. During the embedding steps, spheroids subsections were placed in a  
423 polypropylene vials and collected using a glass capillary with a sealed tip. Before polymerization,  
424 each piece was transferred on to a 22x22 mm coverslip and placed in vertical position for 2 hours.  
425 After that, the resin excess was washed out with absolute ethanol for 3 seconds, and then dried  
426 with filter paper. In some cases, this wash was not necessary (depending on the size of the  
427 organoid) due the low viscosity of the resin. In fact, as shown our recent work<sup>[17]</sup>, the choice of  
428 resin for the embedding is a crucial point for 3D specimen embedding. All coverslips with  
429 spheroids were collected on a silicon mold and put in the oven for the polymerization process at  
430 70°C overnight. In order to evaluate the possible volume change of spheroids during the ROTO-  
431 UTP protocol, a group of 12 intact spheroids prepared with the aforementioned protocol. At the  
432 each cutting step, brightfield imaging (16X magnification) was performed and the total area of the  
433 spheroid was calculated. As shown in the graph, there are no relevant differences in the spheroids'  
434 area across all steps of the ROTO protocol.

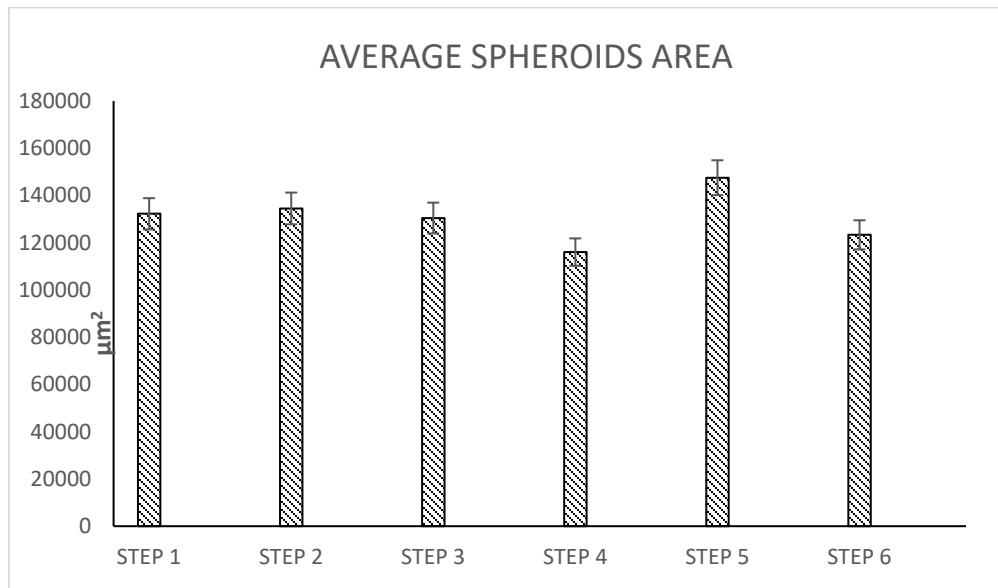
Incubation/washing pieces of spheroids



Pieces of spheroids after polymerization



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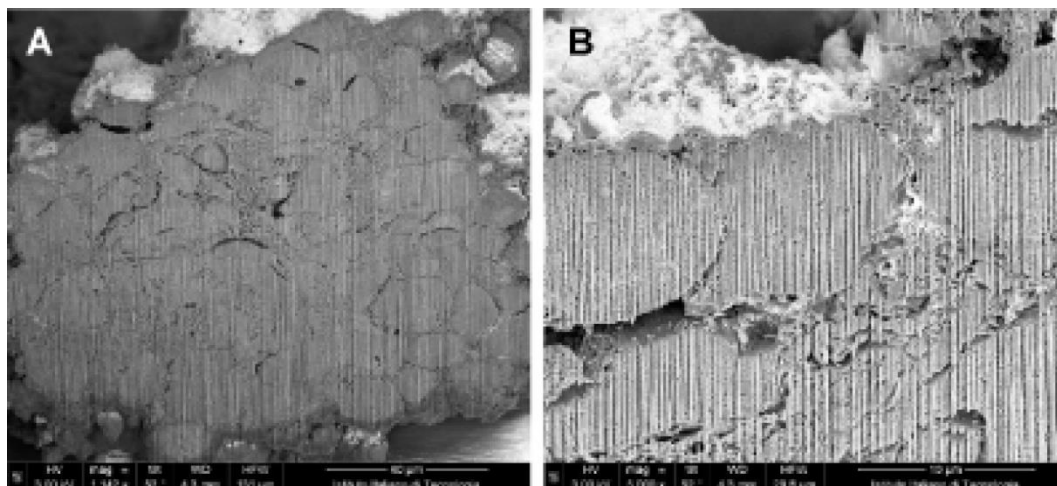


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437

438 **S2-FIB cross sectioning of a spheroid dehydrated *via* CPD.**

439 At an initial observation cells seem to preserve their shapes showing no difference in their external  
440 morphology. However, after the FIB milling (**Figure S2-A**) the intracellular domain has a sponge-  
441 like structure and no ultrastructures are distinguishable.

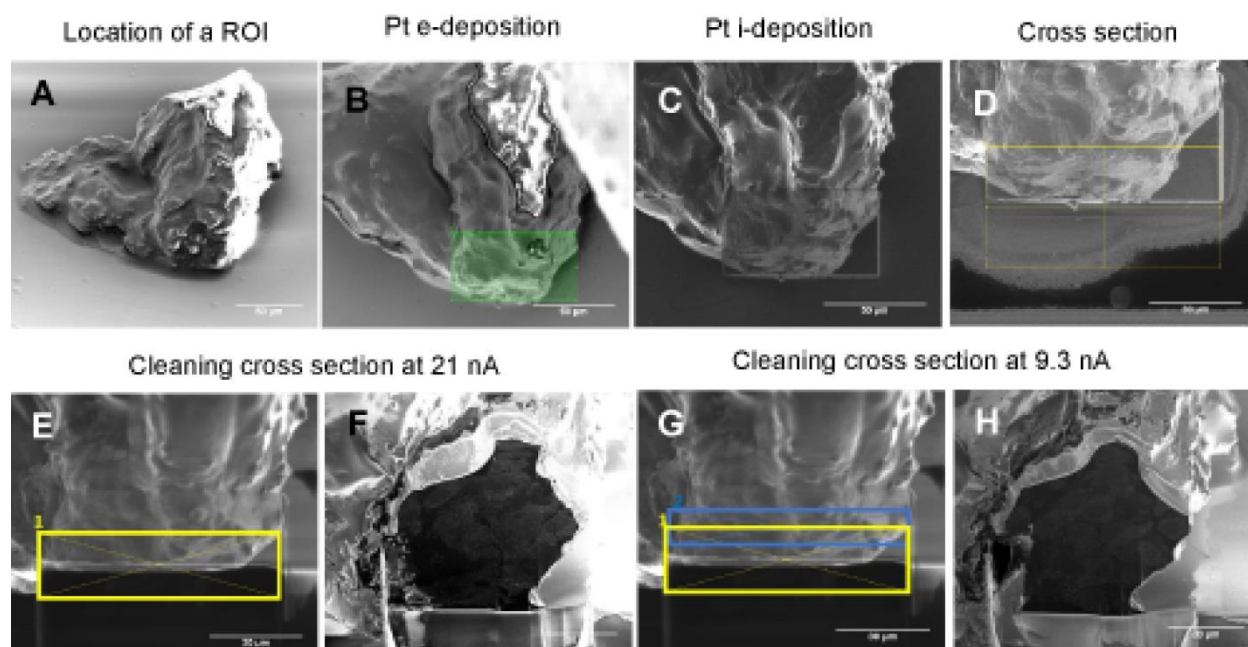


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443 **Figure S2 :** Scanning electron micrographs of FIB cross sections of CPD specimens.

444

445 **S3 – FIB cross sectioning procedure.**



446

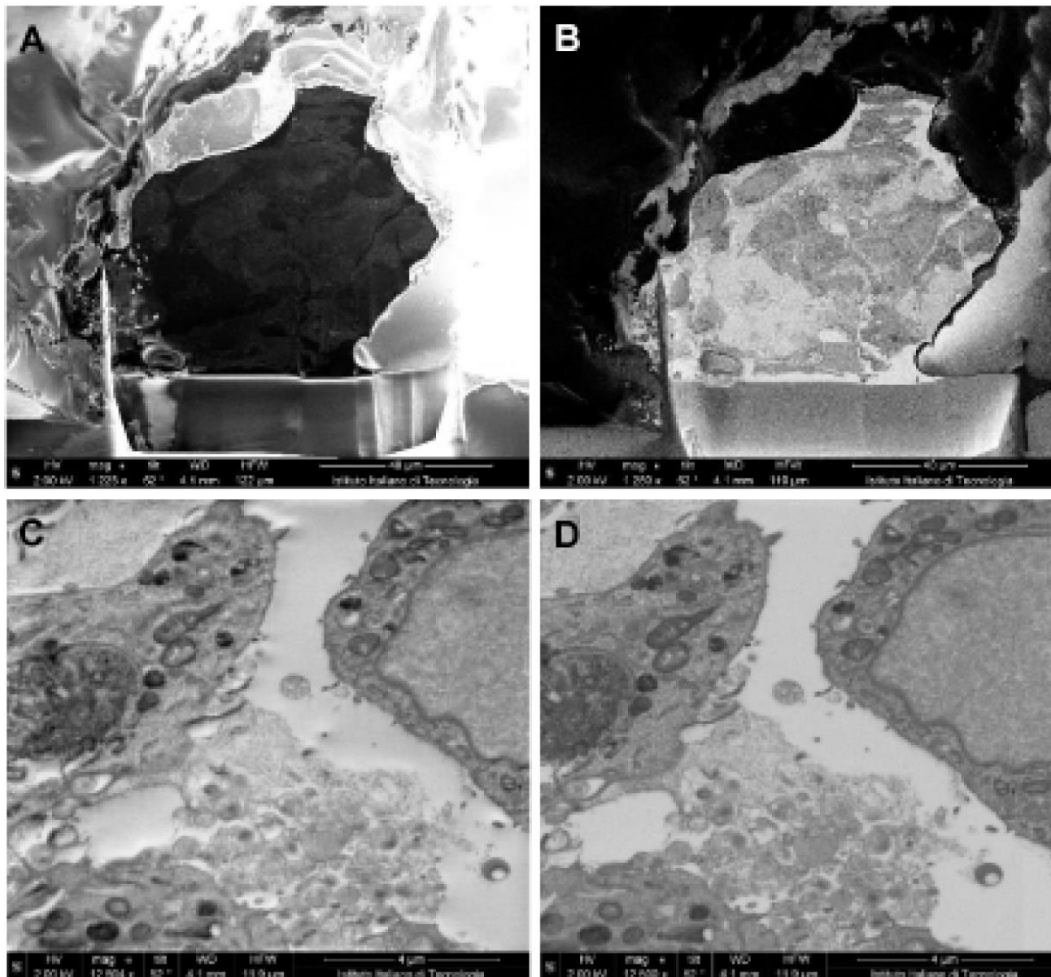
447

448 **Figure S3:** A) location of region of interest (ROI), B) deposition of Pt layer is a rectangular ROI  
 449 of 75  $\mu\text{m}$  by 40  $\mu\text{m}$  (electron beam assisted) C) Pt ion-beam assisted deposition (area of 75  $\mu\text{m}$   
 450 by 40  $\mu\text{m}$ ), D) ion beam milling, E-F) polishing at 21 nA, G-H) polishing at 9.3 nA.

451

452 **S4- Imaging by secondary and backscattered electrons detection.**

453 Secondary electrons are very sensitive to the surface of the material and give mainly information  
454 its 3D morphology (**Figure S4-C**). Instead, backscattered electrons possess higher energy than  
455 secondary electrons thus they are sensitive to the composition of the specimen. Here, the quality  
456 of the images depends on the presence of heavy atoms; an area characterized by heavy atoms  
457 structures appears bright in the backscattered electron image (**Figure S4-B**) and the shape of  
458 borders and internal organelles are clear and well defined (**Figure S4-D**).

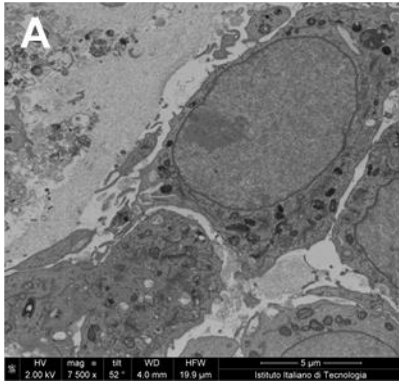


459 **Figure S4** : micrographs acquired with SE A&C) and BSE detectors B&D).

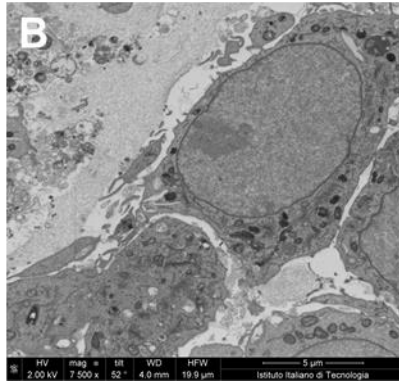
461  
462 **S5 – Image resolution depending on voltage/current in backscattered electrons detection**  
463 **mode.**

464 In **Figure S5**, images of the same area were acquired with the same voltage (2 kV) but increasing  
465 current intensity: 0.20 nA, 0.40 nA, 0.80 nA.

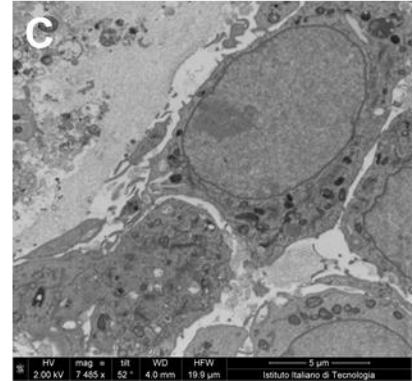
2 kV – 0.20 nA



2 kV – 0.40 nA



2 kV – 0.80 nA



466

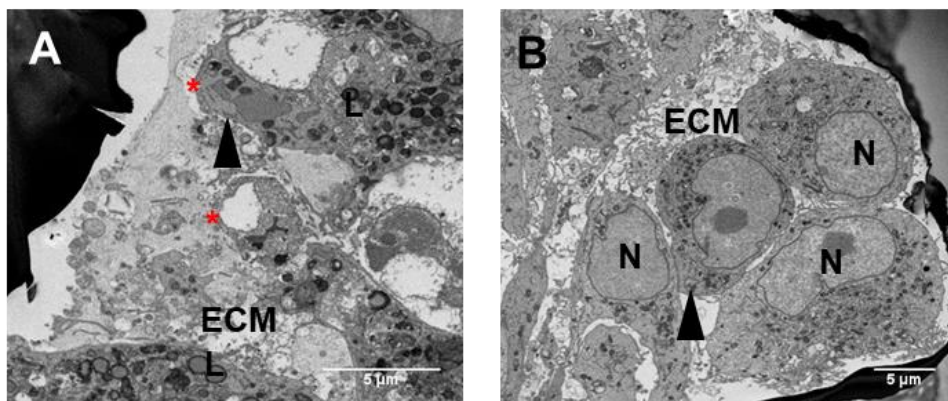
467 **Figure S5:** BSE micrographs of the same area acquired at 2 kV and currents of: A) 0.20 nA B)  
468 0.40 nA C) 0.80 nA.

469

470 **S6- Investigation of ultrastructure in subsection of spheroids obtained by mechanical**  
471 **sectioning after primary fixation in glutaraldehyde (STEP 1).**

472 **Figure S6-A** depicts the outer part of a spheroid while **Figure S6-B** its inner domain. There are  
473 no appreciable damage in organelles or membrane. In fact nuclei, mitochondria, lysosomes, are  
474 well preserved, but the cells appear compressed in their outer domains as they underwent  
475 mechanical stress during the cut.

476



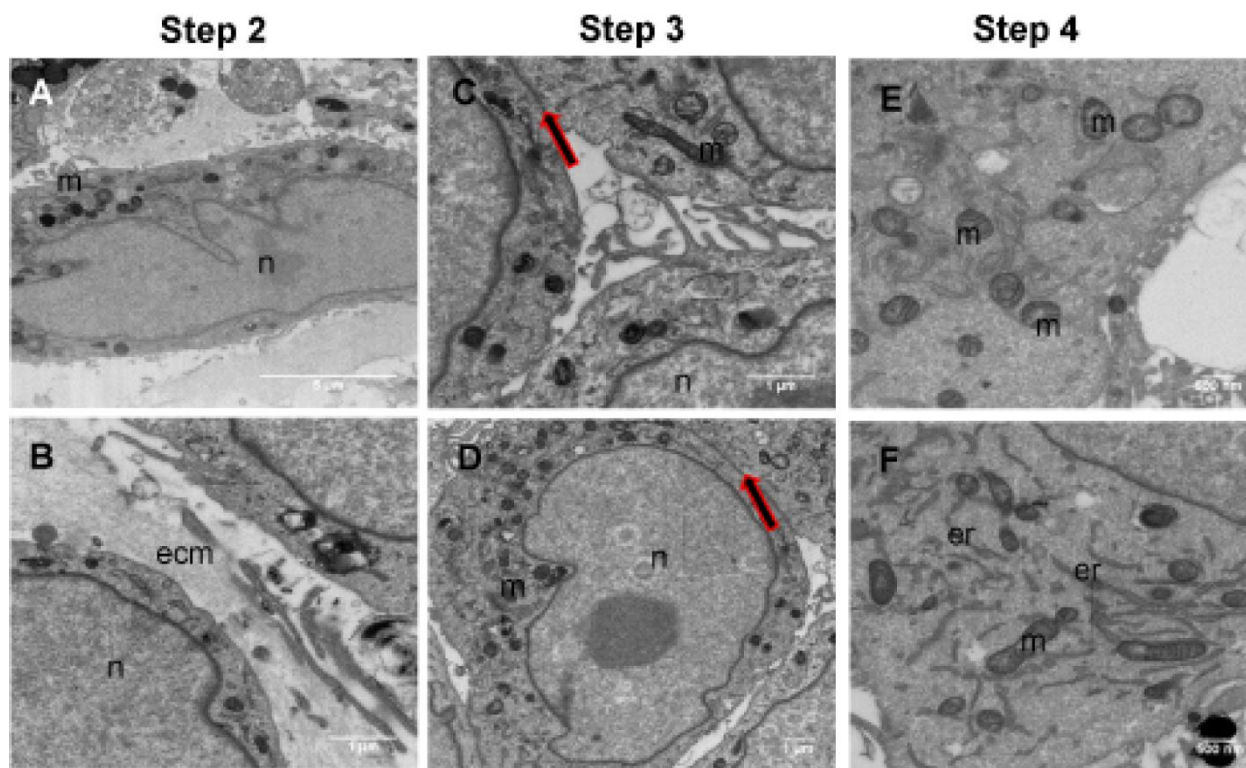
477

478 **Figure S6:** SEM-FIB cross section of U87 derived spheroids after ROTO/UTP protocols at cutting  
479 step 1 (after glutaraldehyde fixation) A) external area of spheroids with evidence of cracks (red  
480 star), B) inner area of spheroids. N: nuclei, ECM: extracellular matrix, L: lysosomes

481

482 **S7- Investigation of ultrastructure in subsection of spheroids obtained by mechanical**  
483 **sectioning at STEP 2, STEP 3, STEP 4.**

484 To investigate the possible mechanical damage due the sectioning of spheroids, SEM/FIB cross  
485 sections of tree intermediate cutting step are reported after treatment with osmium tetroxide (step  
486 2), after tannic acid (step 3) and before polymerization (Step4). In all micrographs, the cell  
487 ultrastructure is well preserved: the cell-cell junctions are evident, and the cytoplasmic organelles  
488 are not eradicated or damaged. In fact the cytoplasm is homogenous, the plasma membrane is  
489 intact, and some organelles, i.e. mitochondria or nuclei, show their peculiar morphology.

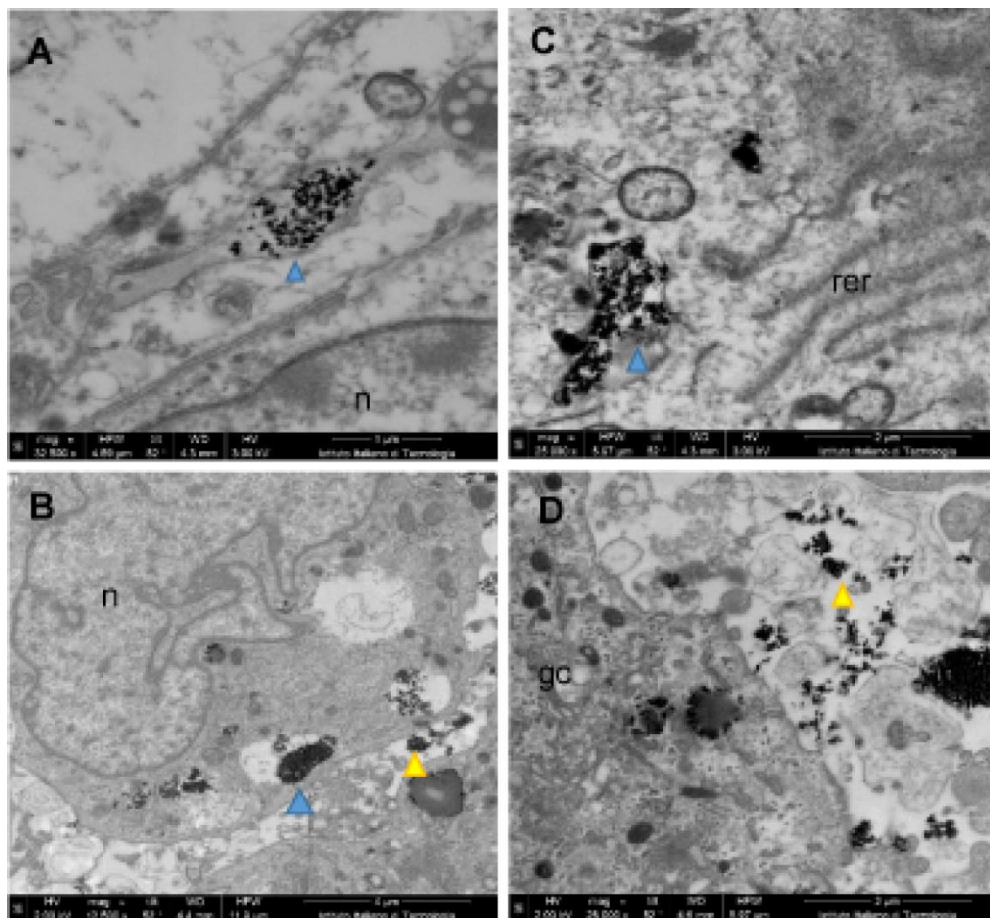


490  
491 **Figure S7:**SEM-FIB cross sections of U87 spheroids treated with ROTO protocol: A,B) after  
492 osmium tetroxide cutting (step2); C,D) after tannic acid cutting (Step 3); E,F) after Spurr's resin  
493 embedding, before polymerization cutting (step 4). Cell-cell junctions are preserved (red/black  
494 arrow), mitochondria (m) crests are definite. Also endoplasmic reticulum is well appreciable  
495 (er)and extra cellular matrix is compact.

496

497 **S8- Internalization of nanovectors in to spheroids.**

498 To characterize the internalization of nanovectors at nanometric resolution, spheroids were  
499 incubated with LMNVs. SEM/FIB cross section were acquired and the spheroid/nanovector  
500 interface was investigated. As shown in **Figure S8**, LMNVs are internalized through endocytosis  
501 in vesicles or vacuoles (blu arrows); nanovectors are also found free in cytoplasm (yellow arrow).  
502



503  
504 **Figure S8:** U87 derived spheroids after ROTO and UTO protocol previously incubated with  
505 LMNVs. Nanovectors are detected in vacuoles (blu arrow) inside the cell and/or free in cytoplasm  
506 (yellow arrows) n: nuclei, gc: Golgi complex, rer: rough endoplasmic reticulum.