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1 **Title:**

2 An original methodology to study polymeric nanoparticle-macrophage interactions: nanoparticle
3 tracking analysis in cell culture media and quantification of the internalized objects

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17 **Abstract**

18 Poly(lactic acid) (PLA) and poly(lactic-*co*-glycolic acid) (PLGA) are among the most employed
19 (co)polymers for the preparation of drug nanocarriers for the treatment of cancer and infectious
20 diseases. Before considering any clinical use, it is necessary to understand the interactions
21 between polymeric nanoparticles (NPs) and their physiological environment, especially immune
22 cells. Here, we propose a simple, yet precise method to assess NPs internalization kinetics in
23 macrophages, based on the direct analysis of the cell culture media after different incubation
24 times. The proof of concept is given here by using fluorescent PLGA NPs. Nanoparticle tracking
25 analysis (NTA) was a method of choice, enabling detecting each individual NP and analyzing its
26 trajectory while in Brownian motion. As compared to dynamic light scattering (DLS), NTA
27 enabled a more precise determination of NP size distribution. The uptake process was rapid: in
28 one hour, around a third of the NPs were internalized. In addition, the internalized NPs were
29 visualized by confocal microscopy. The fluorescent cellular stacks were analyzed using a freely
30 available macro for ImageJ software, Particle_In_Cell-3D. The internalized objects were
31 localized and counted. This methodology could serve for further studies while analyzing the
32 effects of NPs size, shape and surface properties on their interaction with various cell lines.

33 **Keywords:** polymer nanoparticle; poly(lactic acid); size distribution; cellular uptake;
34 nanoparticle tracking analysis; dynamic light scattering; confocal microscopy

35 **1. Introduction**

36 Drug nanocarriers are able to efficiently incorporate, protect towards degradation and ferry active
37 molecules from the administration site to diseased organs, tissues and cells. They are gaining
38 increasing interest for the treatment of severe diseases such as cancer and multiresistant
39 infections (Zhao et al., 2019). While liposomes, discovered early in the 1960s, were the first
40 nanotechnology-based drug delivery system (Finean and Rumsby, 1963), a plethora of other
41 nanocarriers have been developed, including polymeric, hybrid organic-inorganic or metal
42 nanoparticles (NPs), micelles, nanogels, dendrimers, carbon nanotubes and quantum dots.
43 Recently, several nanotechnologies such as Onpattro and Vyxeos were approved by the Food and
44 Drug Administration (FDA), and nowadays, the NP-based vaccines represent a cornerstone in the
45 fight against the COVID-19 pandemic (Jones et al., 2019; Sharma et al., 2021).

46 In particular, polymeric materials have been studied and engineered for more than fifty years,
47 with applications ranging from resorbable sutures and orthopedic implants to
48 nano(micro)particles. The biocompatible and biodegradable poly(lactic acid) (PLA) and
49 poly(lactic-*co*-glycolic acid) (PLGA) are nowadays among the most employed synthetic
50 polymers used to produce drug nanocarriers (Zielińska et al., 2020). A plethora of drug-loaded
51 NPs were produced by various methods aiming to control the main parameters which govern the
52 NPs' *in vivo* fate: size distribution, shape, surface charge and composition. Thus, NPs with
53 increased blood circulation times and targeting abilities were prepared, depending on the
54 application they were destined to (Gref et al., 1994; Ulbrich et al., 2016). The main preparation
55 methods of PLA/PLGA NPs are nanoprecipitation and emulsification-solvent evaporation.

56 Developed in 1989 by Pr. Fessi and his team (Miladi et al., 2016), nanoprecipitation is regarded
57 as a quite simple and reproducible technique that allows the obtaining of submicron-sized

58 polymeric particles. It consists in adding in a drop-by-drop fashion, in an aqueous solution, a
59 polymer in a water-miscible solvent. This results in the immediate precipitation of the polymer
60 under the form of NPs, based on solvent displacement. In emulsification-solvent evaporation, the
61 polymer is dissolved in a volatile solvent non-miscible with water, and an emulsion is formed
62 with an aqueous solution. The droplet size is generally reduced by sonication and the solvent is
63 finally removed. By adjusting the preparation parameters, both NP preparation processes have the
64 potential to be tuned to yield NPs with specific characteristics while maintaining reproducible
65 results (Legrand et al., 2007; Hernández-Giottonini et al., 2020).

66 Upon intravenous administration, NPs interact with cells of the immune system (such as
67 macrophages) which readily recognize them as foreign and withdraw them from blood
68 circulation. NP-macrophage interactions depend on the size and shape of the NPs (Nicolette et al.,
69 2011; Hoshyar et al., 2016; Rattan et al., 2017; Boltnarova et al., 2021). In view of developing
70 medical applications, the fast-growing field of nanomedicine is in need for reliable and
71 convenient methodologies to precisely assess the NPs' size distribution and to investigate their
72 interaction with cells.

73 Nowadays, several techniques are available to determine the NPs' size distribution. The most
74 widespread ones are: i) dynamic light scattering (DLS), the most common and user-friendly
75 method, which allows to estimate the mean NPs' hydrodynamic diameter and polydispersity, but
76 can lead to size overestimations and difficulties in discriminating sub-populations of polydisperse
77 NPs samples (Filipe et al., 2010), and ii) transmission electron microscopy (TEM) and atomic
78 force microscopy (AFM), which allow for an excellent resolution and a precise evaluation of the
79 NPs' diameters, but require a sometimes complex and time-consuming sample preparation, as
80 well as the investigation of large enough NPs populations to enable statistically relevant results
81 (Hoo et al., 2008; Chicea, 2012). In the case of PLA/PLGA NPs, cell internalization processes

82 are generally studied by confocal microscopy, with the aim of comparing NPs formulations, and
83 without quantitative analysis. For quantitative investigations of the internalized amounts, labeled
84 NPs (mostly radioactive ones) are used (Gref et al., 2003; Wu et al., 2020).

85 In this context, we propose here a straightforward method to both accurately characterize NPs
86 size distribution and quantify their interaction with cells. Nanoparticle tracking analysis (NTA)
87 was employed as a means to characterize NPs and investigate their stability in complex media
88 mimicking physiological conditions, as well as to track and quantify their internalization inside
89 immune cells. This method relies on dynamic light scattering to analyze individual NPs'
90 trajectories in their Brownian motion. Advantageously, NTA also allows determining NPs
91 concentration within the sample and possible aggregation events. So far, NTA was only used for
92 the sole purpose of characterizing NPs, like DLS. NTA was sometimes employed to monitor the
93 excretion of exosomes collected from cell cultures (Soo et al., 2012; Zheng et al., 2012), but
94 never yet to study NP-cell interactions.

95 The novelty here is that NTA is used to assess the polymeric NPs internalization kinetics, which
96 has, to our knowledge, never been described before. The proposed method is simple and
97 reproducible. It consists in incubating the NPs with macrophages for different times, harvesting
98 the cell culture media and directly analyzing it by NTA, after appropriate dilution. Thus, the
99 concentration of the NPs in contact with the cells could be accurately determined at each time
100 point. By difference, it was possible to determine the fraction of cell-interacting NPs. To allow
101 the study, the PLGA NPs were labelled with rhodamine (Rho) covalently grafted onto the
102 polymer chain ends, to avoid any leakage during the experiments. Furthermore, to complement
103 these studies, confocal experiments were carried on allowing visualizing NPs and NP aggregates
104 within the cells or at their surface. Particle_In_Cell-3D, an ImageJ macro specially designed for
105 this purpose, was used to count the number of fluorescent objects inside the macrophages

106 (Torrano et al., 2013). Advantageously, this approach allowed to highlight large differences in
107 NPs internalization between cells in the same well. In short, we propose a precise and user-
108 friendly method to not only characterize NPs after interaction with cells, but also to quantify their
109 internalization kinetics.

110 **2. Experimental**

111 **2.1. Materials and reagents**

112 The biodegradable PLGA (50/50) copolymer was a kind gift from Expansorb (Aramon, France).
113 PLA ester-terminated (molecular weight (MW) = 10-18 kDa), PLA acid-terminated (MW = 18-
114 24 kDa), rhodamine B (Rho), polyvinyl alcohol (PVA) (88% hydrolyzed), piperazine, trimethyl
115 aluminum (AlMe_3), 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)
116 carbodiimide (EDC), calcium chloride (NaCl), potassium chloride (KCl), calcium chloride
117 (CaCl_2), magnesium chloride (MgCl_2), potassium hydroxide (KOH), dichloromethane (DCM)
118 and RBS 35 concentrate were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).
119 Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified Eagle medium (DMEM),
120 fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco (Illkirch,
121 France). Ethanol 96% and acetone 100% were purchased from VWR Chemicals (Fontenay-sous-
122 Bois, France). HEPES was purchased from Acros Organics (Illkirch, France). Paraformaldehyde
123 (PFA) 16% was purchased from Electron Microscopy Sciences (Nanterre, France). CellMask
124 Deep Red was purchased from Invitrogen (Illkirch, France). Pure water used in all experiments
125 was filtered (18.4 M cm) by a Milli-Q system (Millipore, Milford, MA, USA).

126 **2.2. Synthesis of PLA-Rho**

127 PLA grafted with Rho at the terminal carboxyl chain end (PLA-Rho) was synthesized by
128 adapting a previously described method (Nguyen and Francis, 2003; Pancani et al., 2018).
129 Briefly, 11.3 mL of a 2 M solution of AlMe₃ in toluene were added to 17.5 mL of a 0.22 mg/mL
130 solution of piperazine in DCM. The reaction mixture was refluxed for 24 h at 50°C with Rho (0.5
131 mg/mL) under inert atmosphere. The obtained Rho-piperazine amide was extracted, purified and
132 dried. 5 mg of Rho-piperazine amide were then reacted with 50 mg of PLA acid-terminated,
133 using DMAP as catalyst and EDC as coupling agent. The obtained Rho-PLA was extracted,
134 purified and dried.

135 **2.3. Preparation of NPs by emulsification-solvent evaporation**

136 An aqueous solution of PVA (0.5% w/v) was prepared by dissolving 150 mg of PVA in 30 mL of
137 Milli-Q water. In order to prepare an aqueous suspension of NPs (15 mg/mL), 60 mg of PLGA
138 were solubilized in 1.5 mL of DCM in a glass vial under magnetic stirring. Then, 4 mL of the
139 PVA solution were added in the vial, followed by vortexing for 20 s to form a coarse emulsion. A
140 fine emulsion was obtained by sonication for 90 s (20% of power), using a sonicator probe
141 (Sonopuls HD 2070, BANDELIN Electronic GmbH & Co, Berlin, Germany). The size of the oil
142 droplets was further reduced by a second sonication step, in an ice bath (30 s, 10% of power).
143 The solvent (DCM) was evaporated overnight under gentle magnetic stirring. Fluorescent NPs
144 were prepared by the same method, by adding 0.2 mg PLA-Rho in the PLGA DCM solution.

145 **2.4. Preparation of NPs by nanoprecipitation**

146 To prepare NPs by nanoprecipitation, 75 mg of PLGA were first solubilized in 1 mL of acetone
147 in a glass vial. Then were added 0.2 mg of PLA-Rho from a stock solution of PLA-Rho, and the
148 resulting solution was diluted with acetone to a final polymer concentration of 12.5 mg/mL.

149 After, 100 μL of this polymer solution were added drop by drop in 1 mL of PVA aqueous
150 solution (0.5% w/v) under magnetic stirring (650 rpm). NPs formed immediately and the
151 suspension was maintained under stirring until complete evaporation of acetone.

152 **2.5. NPs characterization**

153 Three different techniques were used to determine the morphology and the size distribution of the
154 NPs. First, TEM investigations were performed using a JEOL JEM-1400 microscope operating at
155 80 kV. To do so, 4 μL of NP suspensions (concentration ranging between 1 and 15 mg/mL) were
156 deposited onto copper grids covered with a formvar film (400 mesh) for 60 s. The samples were
157 then stained using 2% phosphotungstic acid for 30 s. The excess liquid was blotted off using filter
158 paper, and the grids were dried before observation. Images were acquired using a post-column
159 high-resolution (9 megapixels) high-speed camera (RIO9, Gatan) and processed with ImageJ.

160 The NPs' mean hydrodynamic diameter was also determined by DLS using a Malvern Zetasizer
161 (Nano ZS90, Malvern Panalytical, Worcestershire, UK), with an equilibration time of 120 s. The
162 analyzed samples were diluted using Milli-Q water, aiming for a concentration range of 10 to 100
163 $\mu\text{g/mL}$, and the mean diameters were reported as Z_{Average} (nm) \pm standard error (SE), with a PDI
164 lower than 0.2. All experiments were performed in triplicate.

165 The NPs' average hydrodynamic diameter and concentration were also evaluated by NTA
166 (NanoSight LM10, Malvern Panalytical, Worcestershire, UK). 1 mL of the sample was
167 introduced in the device's chamber, with a concentration range of 1 to 5 $\mu\text{g/mL}$, which
168 corresponds to an approximate concentration of 10^8 objects/mL. Once the NPs were exposed to a
169 laser beam, the light scattering and Brownian motion were both recorded on video and analyzed,
170 which allowed to characterize the NPs individually. For each sample, five videos of 60 s each

171 were recorded. In order to be certain that only NPs would be visualized and analyzed, a
172 fluorescence filter was applied all through the recordings.

173 **2.6. NPs intracellular internalization using NTA and confocal microscopy**

174 The J774A.1 cell line was obtained from ATCC and cultured at 2×10^5 cells/mL, using DMEM
175 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO₂.
176 When studying NPs internalization, 5×10^5 cells in 1 mL of medium were deposited on cover
177 glasses at the bottom of 6-well plates. Cover glasses had been previously sterilized by immersion
178 in an aqueous solution of RBS (2%) at 50°C before being dipped in 90% ethanol and dried under
179 a biological hood before use. Then, cells were incubated at 37°C and 5% CO₂ overnight. The
180 next day, the medium was removed and replaced with 1 mL of medium containing 4×10^{10} NPs.
181 After different incubation times (from 1 to 24 h), supernatants were harvested and diluted in
182 Milli-Q water before being analyzed by NTA.

183 HEPES buffer (20 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, pH
184 adjusted to 7.4 with KOH) was prepared for the analysis of cells on cover glasses, in order to
185 ensure pH homeostasis and avoid osmotic pressure changes. Cover glasses were placed in 300 µL
186 of CellMask Deep Red (1 X, diluted in HEPES buffer), a cell membrane marker, for 5 min at
187 37°C. A fixation solution comprising HEPES buffer and PFA (3.75%) was prepared. The
188 membrane marker was removed and cover glasses were placed in 1 mL of the fixation solution
189 for 5 min at 37°C. The fixation solution was then removed and cover glasses were cleaned three
190 times in DPBS.

191 Confocal microscopy was performed immediately with a Leica TCS SP5 device (AOBS,
192 Mannheim, Germany) equipped with a Leica objective (63×/1.4 NA, oil immersion). For Rho

193 imaging was used the 514 nm line of a continuous Ar laser, and for CellMask Deep Red imaging,
194 the 633 nm line of a continuous He-Ne laser. Images were produced in direct slow scanning
195 sequential mode (400 Hz) and the fluorescence was collected in the spectral range from 520 to
196 600 nm for Rho, and from 640 to 680 nm for CellMask Deep Red. The resolution of the confocal
197 images was 512×512 pixels, recorded on 8 bits with different zoom values and a pinhole size of 1
198 airy. Their size in μm depended on the zoom (between 2 and 4). Images stacks were acquired in
199 order to observe all the layers of the cells with a step of 0.130 μm (around a hundred images were
200 necessary). The stacks were then analyzed using ImageJ, with a macro (Torrano et al., 2013)
201 aiming to create cell masks, 3D representations of cells from the stacks, and to quantify
202 intracellular NPs.

203 **3. Results and discussions**

204 **3.1 DLS and NTA: characteristics and assets**

205 Three simultaneous goals were pursued here: i) accurately determine the size distribution of the
206 polymeric NPs; ii) study their stability in biological medium, and iii) quantify their
207 internalization kinetics inside macrophages. The NPs' size distribution is commonly determined
208 by DLS, a well-known and non-destructive method schematized in **Fig. 1. A**. Briefly, the
209 principle of this method consists in illuminating a suspension of NPs with a laser source. The
210 intensity of the light scattered by the NPs in their Brownian motion is measured at a given angle
211 (typically 90°) allowing to estimate the mean hydrodynamic diameter and size distribution of the
212 sample. Through its simplicity of use, DLS is a widespread method; however, it only allows to
213 study populations of NPs, with no possibility of observing individual objects. Thus, in the case of
214 a relatively polydisperse NP population, DLS is not well adapted to discriminate sub-populations.

215 Moreover, the largest NPs scatter more light than the smaller ones, leading to an overestimation
216 of the mean hydrodynamic diameters (Filipe et al., 2010; Varenne et al., 2016).

217 Addressing the challenge of estimating accurately the size distribution of a polydisperse NP
218 sample thus requires a method which would allow to study populations of NPs and individual
219 objects at the same time (Chaupard et al., 2021). Microscopies such as TEM and AFM enable to
220 determine the size of isolated NPs with a high resolution, but besides requiring an experimented
221 user and a sometimes complex sample preparation, it is cumbersome to analyze populations large
222 enough to obtain representative results.

223 In this context, NTA is an interesting alternative. The NPs in the device's chamber are
224 illuminated by a laser beam, which makes them appear as bright spots. This enables to visualize
225 and track the trajectories of individual NPs in their Brownian motion using a classic optical
226 microscope. (**Fig. 1. B**). From the trajectories of thousands of NPs, individual hydrodynamic
227 diameters are calculated using the Stokes-Einstein equation. Contrary to DLS, which analyzes
228 “bulk” samples, NTA allows to reconstitute size distributions from individual NPs measurements.
229 Another advantage is that, considering the analyzed volume is known, NTA allows to calculate
230 the concentration of the sample. Finally, the device can be equipped with fluorescence filters,
231 which allow to track only the fluorescent objects in the sample. This is an asset in studying NPs
232 in complex environments such as serum-containing cell culture media. A recording without
233 (**Supplementary material 1**) or with a fluorescence filter on (**Supplementary material 2**) is
234 available in the supplementary materials.

235 [Figure 1]

236 NTA and DLS were comparatively used here to determine the NPs' size and to investigate their
237 interactions with macrophages. First, fluorescent PLGA NPs were successfully prepared by two
238 methods, nanoprecipitation and emulsification-solvent evaporation. They were named NPs-P and
239 NPs-E, respectively. The fluorescent dye Rho was covalently grafted onto the polymeric cores, to
240 avoid any leakage during the experiments (Pancani et al., 2018). Both types of NPs appeared
241 round-shaped and with homogeneous sizes when observed by TEM (**Fig. 2**). The images also
242 showed a more polydisperse population of NPs-E (**Fig. 2. B**) than NPs-P (**Fig. 2. A**). After
243 analyzing a hundred objects, the mean diameters were 78 ± 28 nm and 117 ± 50 nm for NPs-P
244 and NPs-E respectively.

245 The suitability of NTA for studying these fluorescent NPs was further investigated. Indeed,
246 knowing that the NPs would be investigated in complex media such as cell culture media, it was
247 important that they would be visible with a fluorescence filter on, so that only them would be
248 detected, and no other parasite object from the medium. Thus, both samples, NPs-P and NPs-E,
249 were analyzed with and without a fluorescence filter.

250 Without the fluorescence filter, NPs-P exhibited a concentration of $4.45 \times 10^8 \pm 2.07 \times 10^7$
251 particles/mL, and a mean hydrodynamic diameter of 171 ± 36 nm. With the fluorescence filter,
252 the concentration was $3.24 \times 10^8 \pm 1.54 \times 10^7$ particles/mL, and the mean hydrodynamic diameter,
253 159 ± 39 nm (**Fig. 2. C**). For NPs-E, without the fluorescent filter, the concentration was
254 $5.53 \times 10^8 \pm 1.38 \times 10^7$ particles/mL, and the mean hydrodynamic diameter, 260 ± 80 nm. With the
255 fluorescence filter, the values were $4.03 \times 10^8 \pm 1.16 \times 10^7$ particles/mL for the concentration, and
256 280 ± 94 nm for the mean hydrodynamic diameter (**Fig. 2. D**).

257 One result to notice is that the size distribution confirms what could be seen on the TEM images:
258 for NPs-P, a very narrow size distribution can be observed, which supports the idea of a
259 monodisperse population. For NPs-E, however, several peaks were recorded, indicating the
260 presence of a quite polydisperse population. Another observation is that with the fluorescent filter
261 on, ~ 27% of the NPs-P and the NPs-E were not detected by the device. Such an observation can
262 be explained by the fact that with the filter on, the image perceived by the camera is generally
263 darker, and thus, focusing on the NPs is more difficult. In this context, in order to optimize the
264 investigations, a higher quantity of Rho could be incorporated into the NPs. However, this partial
265 detection does not seem to have any influence on the mean hydrodynamic diameter evaluation. In
266 short, some objects might go unnoticed, but both NPs-P and NPs-E are efficiently detected and
267 analyzed with the fluorescence filter on.

268 Finally, the question of whether cell culture medium would interfere with NPs detection or not
269 was of utmost importance. NPs-P and NPs-E were suspended in cell culture medium at a suitable
270 concentration for NTA and analyzed with and without the fluorescence filter. With the filter,
271 NPs-P exhibited a concentration of $4.01 \times 10^8 \pm 9.95 \times 10^6$ particles/mL and a mean hydrodynamic
272 diameter of 150 ± 39 nm (**Fig. 2. E**), while NPs-E showed a concentration of $1.83 \times 10^8 \pm$
273 5.48×10^6 particles/mL and a mean hydrodynamic diameter of 250 ± 101 nm (**Fig. 2. F**). For both
274 samples, the size distribution remained the same as in Milli-Q water (indicating that the NPs did
275 not aggregate) (**Fig. 2. C and D**). As a conclusion, fluorescent NPs can be analyzed efficiently by
276 NTA in complex media, provided the adequate fluorescence filter is on. Indeed, without the filter,
277 so many objects were detected that the software could not complete the analysis.

278 [Figure 2]

279 **3.2 Comparison of NTA and DLS results**

280 It is established in the literature that DLS can sometimes overestimate the mean hydrodynamic
281 diameter, if the sample contains aggregates or bigger sub-populations (Filipe et al., 2010). This
282 observation was put to the test with the studied NPs-P and NPs-E samples. On **Fig. 3. A and B**,
283 screenshots taken during the NTA analysis of NPs-P and NPs-E, respectively, can be seen. The
284 recorded trajectories of the NPs can also be observed. On **Fig. 3. C and D**, the results of both
285 NTA and DLS analyses are displayed.

286 A first observation is that for both NPs-P and NPs-E, the mean hydrodynamic diameters
287 calculated by DLS are larger than the ones determined by NTA. Differences as high as 50 nm
288 were observed in the case of NPs-E. The second finding concerns the size distribution recorded
289 by DLS. For NPs-P, the DLS size distribution is narrow, similarly to the NTA one. But for NPs-
290 E, the DLS distribution is much broader and, more importantly, does not reflect the several
291 populations which were detected by NTA (**Fig. 2. D**). These findings show that, despite the fact
292 that DLS is a rapid and user-friendly method, it might overestimate the mean hydrodynamic
293 diameter and, furthermore, not be able to discriminate sub-populations in a polydisperse sample.
294 Finally, NTA appears to be an adequate alternative to TEM and DLS, being able to analyze
295 important NP populations on an individual basis and within a short timeframe.

296 [Figure 3]

297 **3.3 Study of NP internalization kinetics by NTA**

298 This study proposes a use of NTA which, to our knowledge, has never been described in the
299 literature before. The objective was to quantify the internalization of NPs by macrophages in a

300 simple and reproducible manner. For the proof of concept, NPs-E were put to incubate with
301 J774A.1 macrophages for different time periods, from 1 to 24 h. The cell culture media
302 containing the NPs not engulfed or not adhered to the macrophages were recovered. After a
303 suitable dilution with Milli-Q water, NTA experiments were performed with the NP suspensions.
304 The postulate was that all NPs which were not present in the cell culture media had interacted
305 with the macrophages. Therefore, by knowing the initial concentration of NPs incubated with the
306 macrophages at the start of the experiment and by measuring the NP concentration in the
307 collected cell culture media, it was possible to track the NPs' internalization kinetics.

308 **Fig. 4** shows the results obtained by incubation of NPs-E suspensions at an initial concentration
309 of 200 µg/mL with macrophages, and the control experiment, corresponding to the NPs
310 incubation at 37°C in cell culture medium, in the same conditions, but without macrophages. The
311 concentration of NPs-E which were incubated for 24 h in the absence of cells would remain the
312 same. The mean hydrodynamic diameter was of 292 ± 11 nm, similarly to what was observed in
313 Milli-Q water, suggesting that no aggregation occurred (**Fig. 2. D**). In contrast, when the same
314 NPs were incubated with macrophages, the NPs concentration was dramatically reduced,
315 indicating that interactions took place with the macrophages.

316 The internalization process occurred in two phases: a first one, rapid (1 h), during which around a
317 third of the NPs were internalized; and a second one, until the end of the study, during which NPs
318 were internalized in a slower manner. One hypothesis is that the macrophages which were present
319 from the beginning of the experiment internalized a large amount of NPs until reaching
320 saturation, and that cell growth progressively produced new macrophages which continued
321 internalizing the NPs remaining in the supernatants. Another possibility is that NPs progressively
322 sediment in the cell culture media so their concentration in the vicinity of the macrophages

323 increases, leading to further uptake. Of note, the influence of the sedimentation processes on cell
324 uptake were discussed previously (Cho et al., 2011; Yazdimamaghani et al., 2018).

325 [Figure 4]

326 **3.4 Study of NP internalization kinetics by confocal microscopy**

327 Whereas NTA enabled to quantify the NPs internalization process by measuring their decreasing
328 concentration in the cell culture media over time, confocal microscopy is a complementary
329 technique, as it allows to directly visualize the NPs' presence inside the macrophages or on their
330 membrane. As an example, a typical image of a J774A.1 macrophage having been in contact with
331 NPs-E for 2 h is presented in **Fig. 5. A and B**. A large number of NPs (green spots) can be
332 observed inside the cytoplasm as well as on the cell membrane (red) (the full stack of images can
333 be seen in **Supplementary material 3**). A whole 3D reconstruction of the cell can be
334 conveniently obtained by confocal microscopy (rotating reconstructions are available in
335 **Supplementary material 4 and 5**). However, we intended here to go steps beyond by
336 quantifying the number of internalized NPs in each cell. To do so, the free ImageJ macro
337 Particle_In_Cell-3D was updated and used here
338 (https://imagejdocu.tudor.lu/macro/particle_in_cell-3d) (Torrano et al., 2013).

339 Briefly, the program analyzes images stacks where both cell membranes and NPs have to be
340 fluorescently marked in order to create an accurate 3D representation of the cell with the objects
341 inside, and to quantify the said objects. In **Fig. 5. C** is displayed the mask that the macro creates
342 before producing the 3D representation with the help of the ImageJ plugin 3D Viewer (**Fig. 5. D**).
343 The virtual cell can then be rotated and cut open in order to observe the internalized NPs (**Fig. 5.**
344 **E and F**). Thus, when a sufficient number of cells is analyzed, Particle_In_Cell-3D could serve as
345 a statistical tool to quantify, for each time point in a kinetic study, the mean number of NPs

346 internalized by immune cells.

347 [Figure 5]

348 In **Fig. 6. A** can be seen a group of macrophages after 2 h of incubation with NPs-E. Surprisingly,
349 some of them internalized large amounts of objects, while others only internalized a few of them.
350 Particle-In-Cell_3D can serve as a means to prove and support this observation. Indeed, six
351 representative cells were analyzed. In this method, the quantification is based on the total
352 fluorescence (i.e. pixel intensity) of the detected NPs. While for some cells, the total fluorescence
353 intensity was about 3×10^6 (arbitrary unit), for other cells, the measured value for intracellular
354 NPs was around 22×10^6 (arbitrary unit). In short, the macro confirmed that the amount of NPs
355 internalized by single cells greatly vary, with neighboring cells having a difference of up to seven
356 times in the quantity of intracellular objects.

357 In **Fig. 6. B, C, D** and **E** are studied different cells for each time point of the kinetic study. Up to
358 2 h of incubation, internalized NPs appear as sharp and individualized signals. However, the
359 more time passes, the more those signals appear as large and diffuse halos, making it harder to
360 discriminate one from another. Knowing that Rho was covalently bound to the NPs-E, this
361 change probably indicates that NPs degrade inside the macrophages, releasing their Rho label,
362 which produces a diffuse signal around the visualized objects. Possibly, this degradation process
363 is accelerated by the acidic microenvironment inside the lysosomes. In order to study this
364 phenomenon further, it will be necessary to decipher the involved internalization mechanisms.

365 [Figure 6]

366 **4. Conclusion**

367 Fluorescent PLGA NPs were prepared by nanoprecipitation and emulsification-solvent
368 evaporation methods. The latter method gave larger and more polydisperse populations. NTA

369 based on tracking individual NPs was more precise than DLS to identify the various populations
370 within a sample.

371 We have demonstrated the feasibility of an easy-to-use technique for the quantification of
372 polymeric NPs internalization by immune cells, a necessary step in deciphering the interactions
373 between potential drug nanocarriers and the physiological medium. We propose a use of NTA
374 which has never been described before and combine the results to those provided by a freely
375 available ImageJ macro for a precise evaluation of internalization kinetics.

376 This work presents a proof of concept of the method. In further studies, this technique could be
377 used on larger scales to test several formulations of NPs and identify the most suited ones for the
378 use they are destined to. For example, the impact of size, shape, surface charge and coating on
379 internalization kinetics could easily be tested.

380 Besides, this method could also help to decipher the internalization cellular mechanisms involved
381 depending on the analyzed NPs, using for example several internalization inhibitors such as
382 chlorpromazine, nystatin or dynasore. Ultimately, the strategy presented here could be used for *in*
383 *vitro* tests before the *in vivo* evaluation of the NPs formulations.

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390 INBS-04 / FranceBioImaging; ANR-11-IDEX-0003-02 / Saclay Plant Sciences).

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472 Captions to Figures

473 **Fig. 1. A.** Schematic representation of the DLS method. A laser beam is aimed at the sample.
474 When crossing the laser beam, NPs scatter the light, the larger ones more than the smaller ones.
475 The information is treated in order to estimate the mean hydrodynamic diameter of NPs in the
476 sample. **B.** Schematic representation of the NTA method. Dynamic light scattering is also
477 involved as NPs are illuminated by a laser beam. With an optical microscope coupled to a

478 camera, the NPs' trajectories are recorded and analyzed (as depicted on the right), which serves
479 to determine their individual hydrodynamic diameters accurately.

480 **Fig. 2. A and B.** TEM images of NPs-P and NPs-E respectively. **C and D.** NTA profiles of NPs-
481 P and NPs-E in Milli-Q water respectively, with (solid line) or without (dotted line) a
482 fluorescence filter. **E and F.** NTA profiles of NPs-P and NPs-E in cell culture medium
483 respectively.

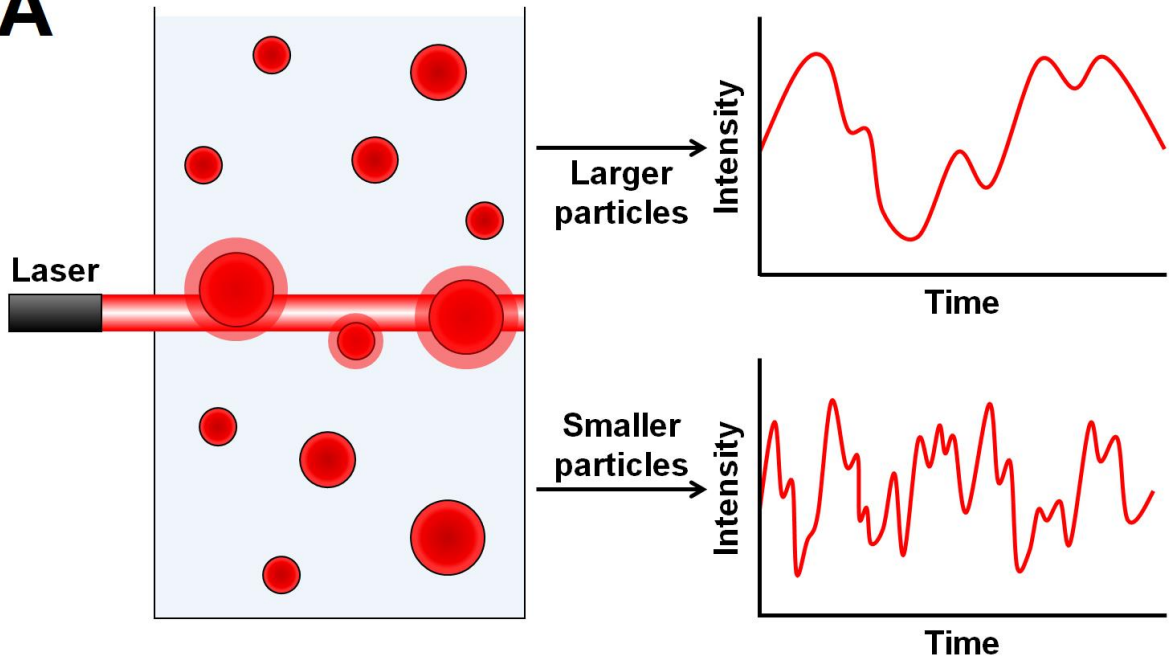
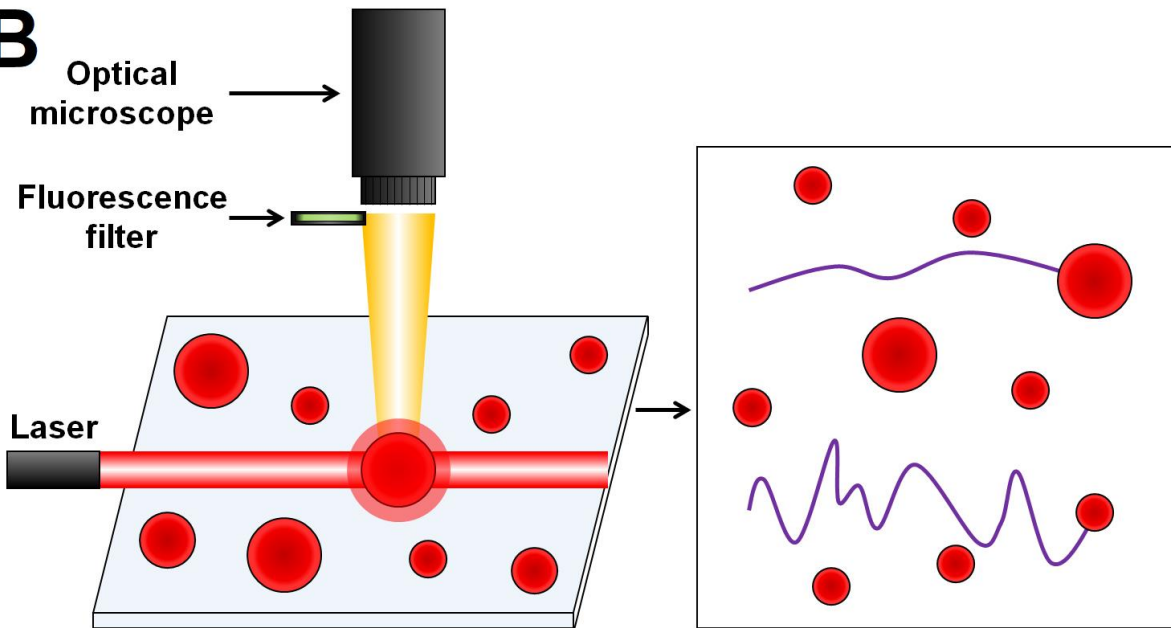
484 **Fig. 3. A and B.** Screenshots taken during the NTA analysis of NPs-P and NPs-E in Milli-Q
485 water respectively. The recorded video is played in the background while the NPs' trajectories
486 (red lines) are analyzed and the size distribution graph is calculated at the same time (blue curve).
487 During the analysis, the graph changes every second, cumulating the information provided by the
488 recorded video. **C and D.** Size distribution graphs comparing the results obtained with NTA and
489 DLS (number distribution) for NPs-P and NPs-E in Milli-Q water respectively.

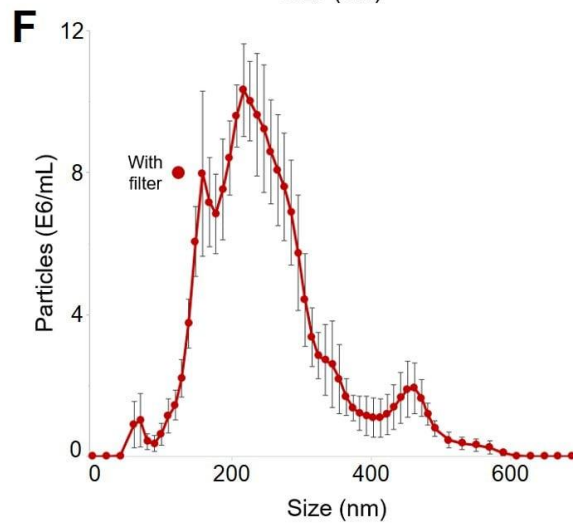
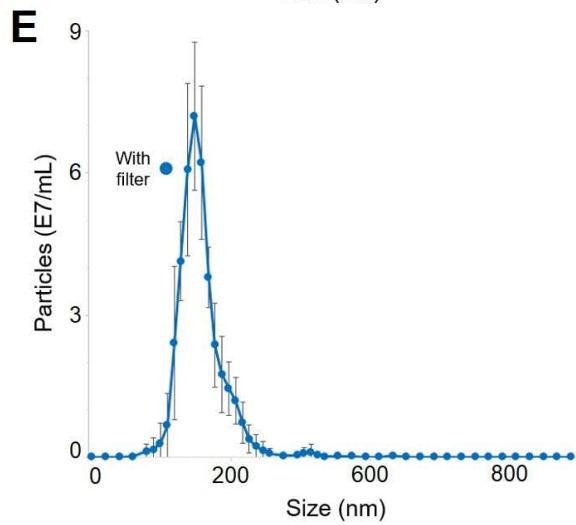
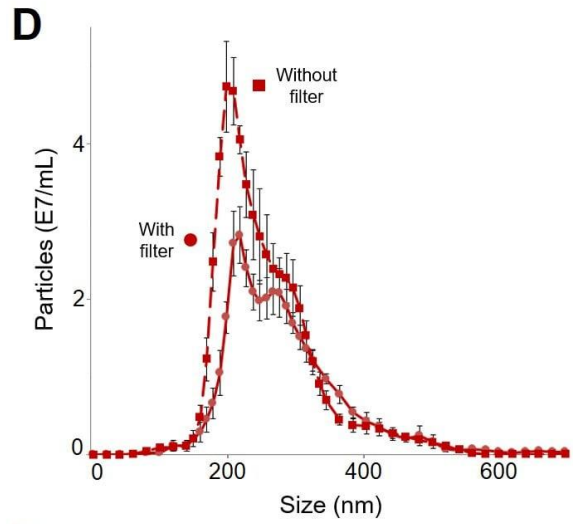
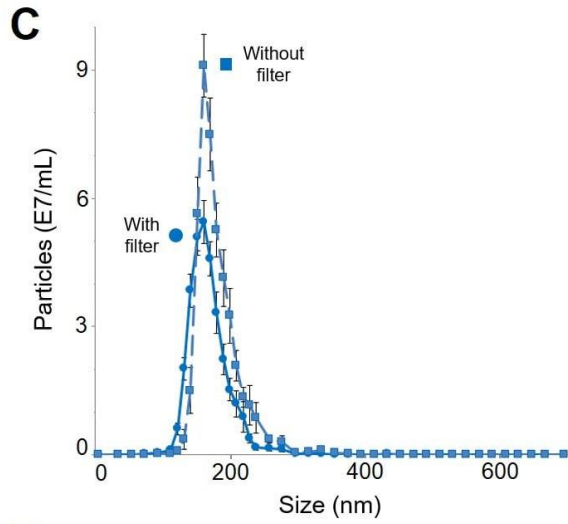
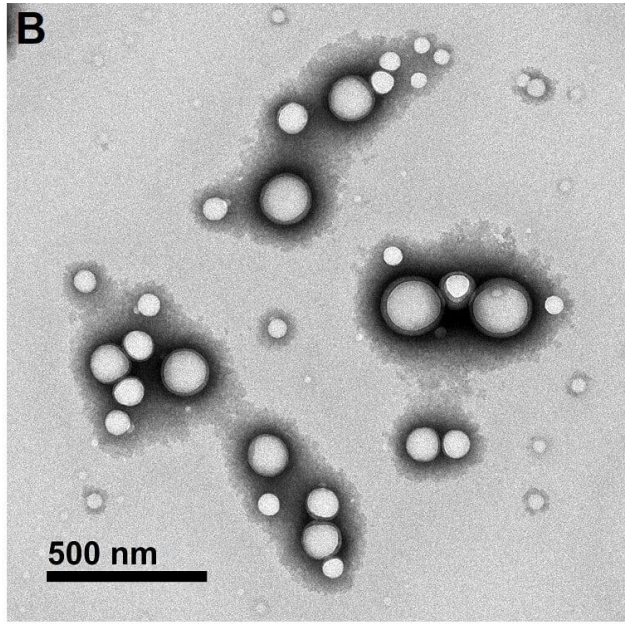
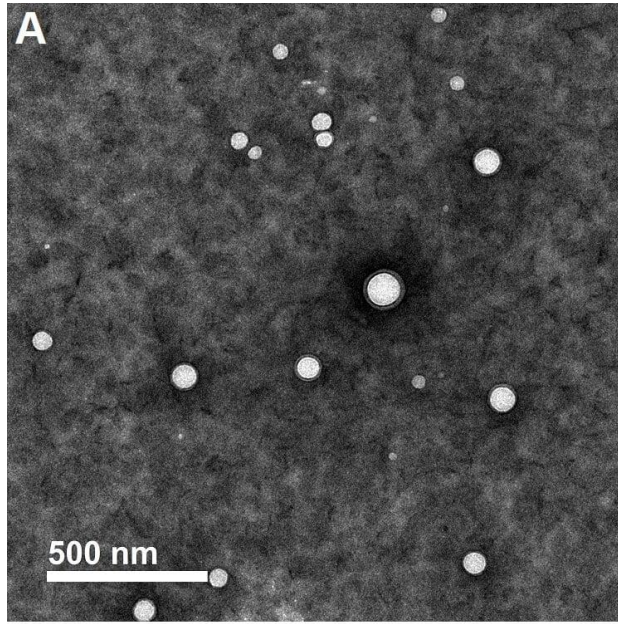
490 **Fig. 4.** Internalization curve of NPs-E inside J774A.1 macrophages (red curve) and control
491 corresponding to NPs-E incubated under the same conditions, but without J774A.1 macrophages
492 (gray curve).

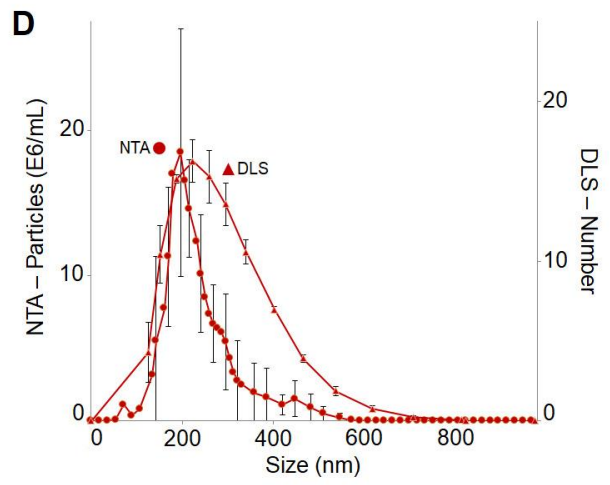
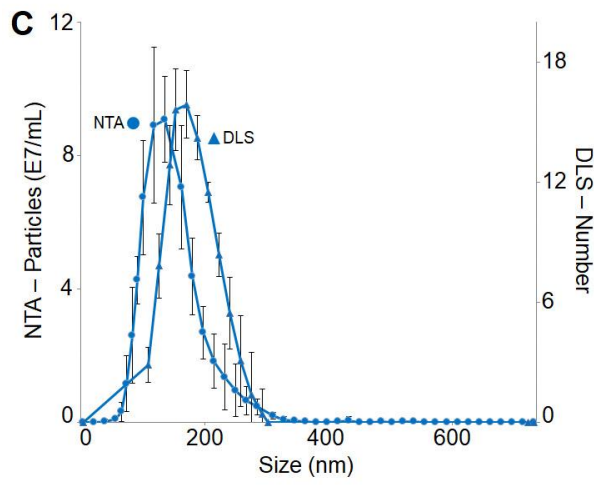
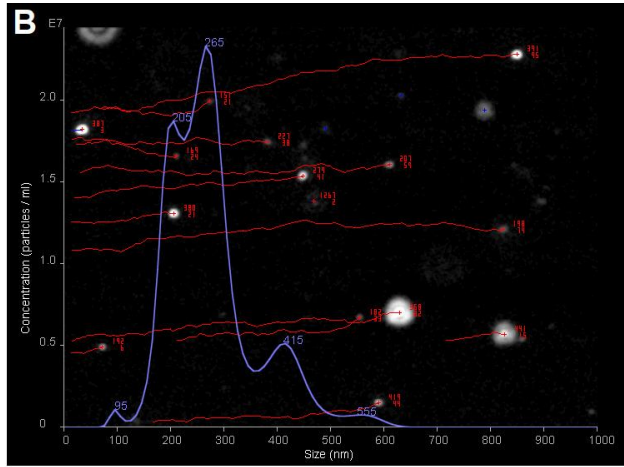
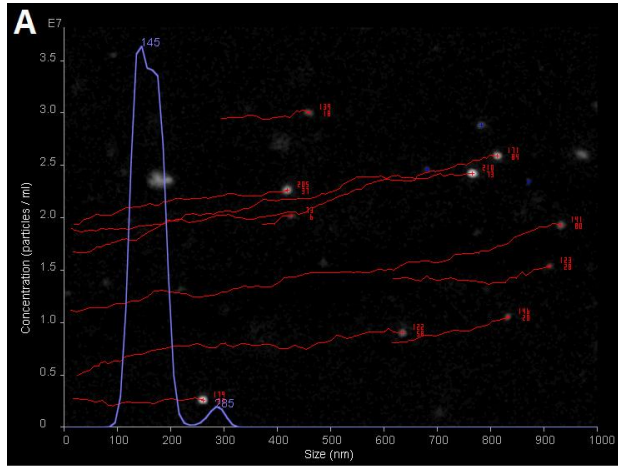
493 **Fig. 5. A and B.** J774A.1 macrophage with internalized NPs-E (2 h of incubation) observed by
494 white light and fluorescence confocal microscopy (cell membrane in red, NPs in green)
495 respectively. **C.** Cell mask created by the macro Particle_In_Cell-3D
496 (https://imagejdocu.tudor.lu/macro/particle_in_cell-3d). **D, E and F.** 3D representation of the
497 macrophage created by the macro, under different angles.

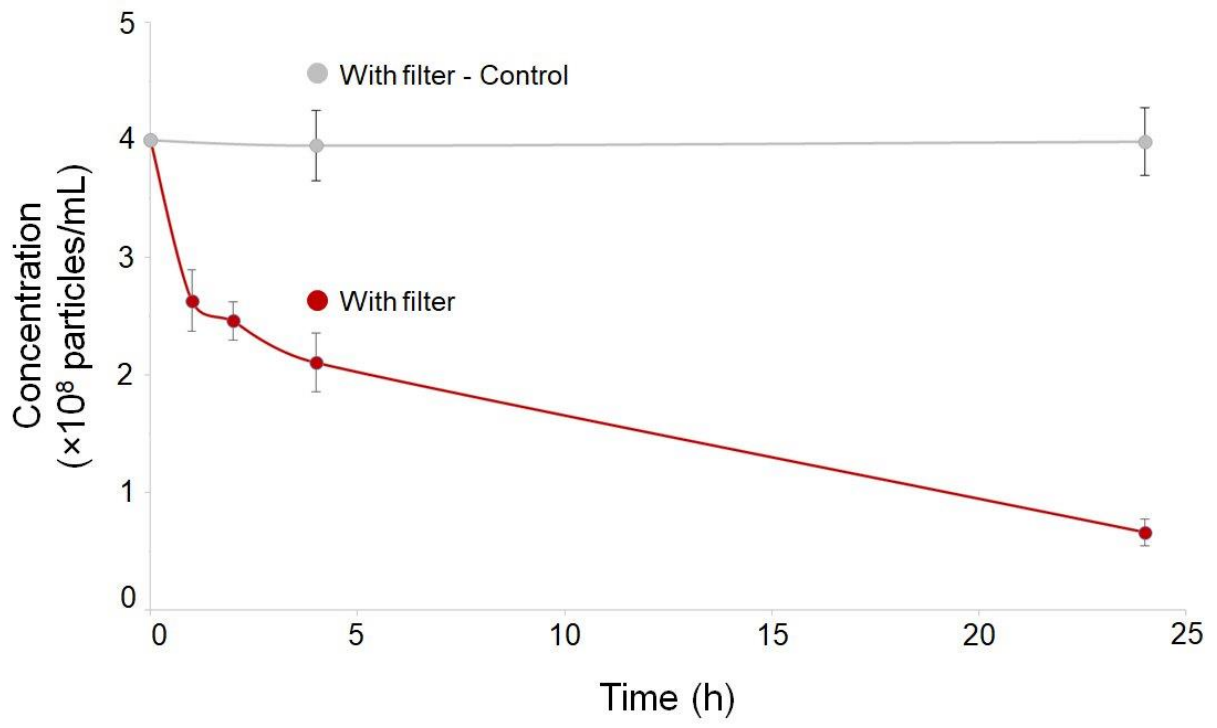
498 **Fig. 6. A.** Group of J774A.1 macrophages with internalized NPs-E (2 h of incubation) observed
499 by fluorescence confocal microscopy (cell membranes in red, NPs in green). **B, C, D and E.**

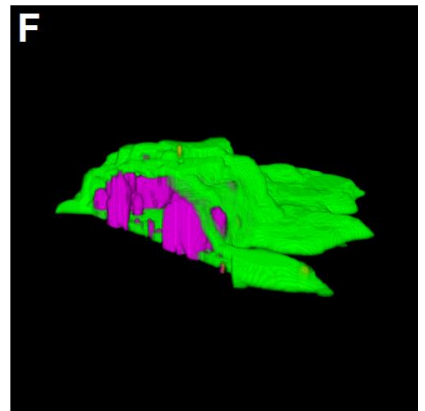
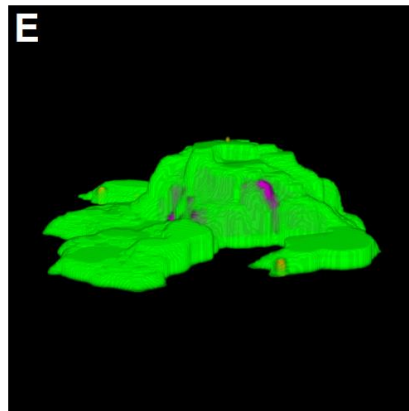
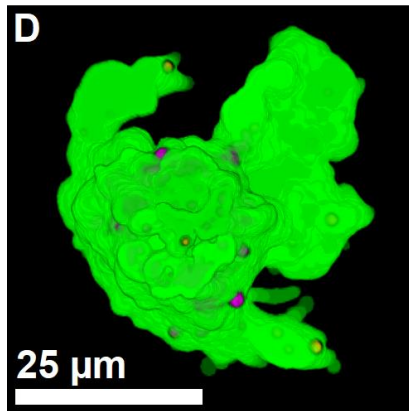
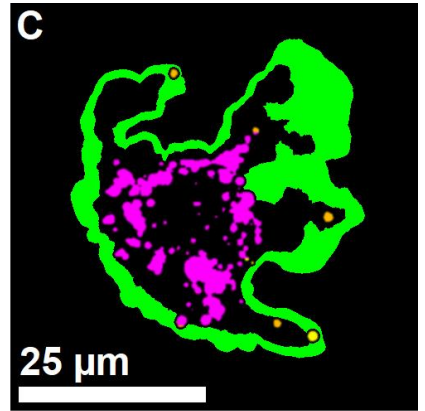
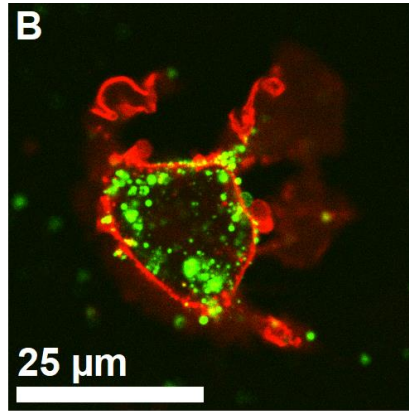
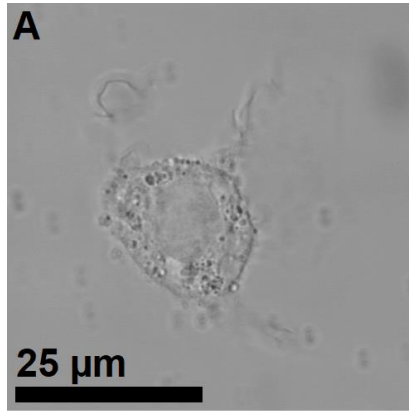
500 J774A.1 macrophages with internalized NPs at different times of incubation (1 h, 2 h, 4 h and 24
501 h respectively).

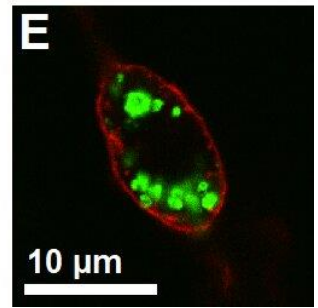
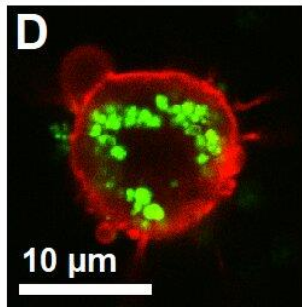
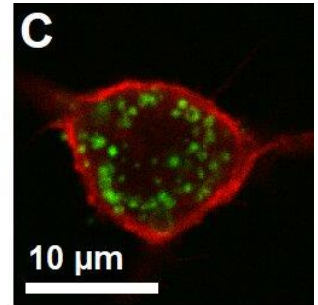
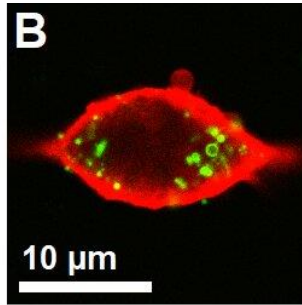
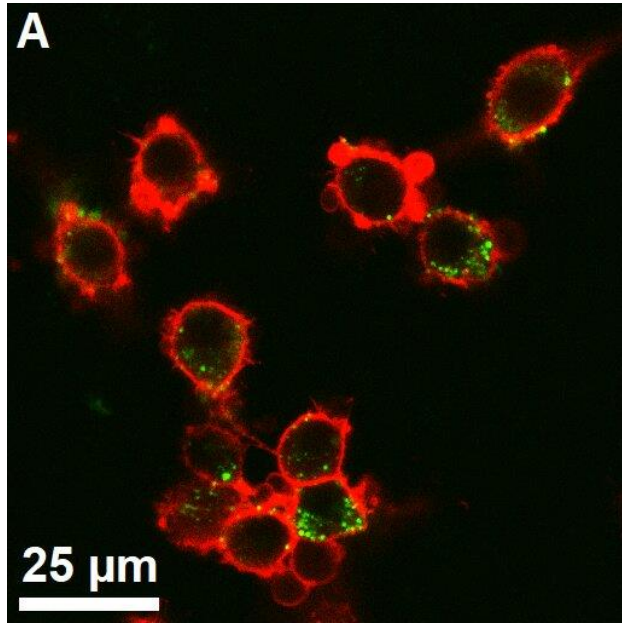
A**B**

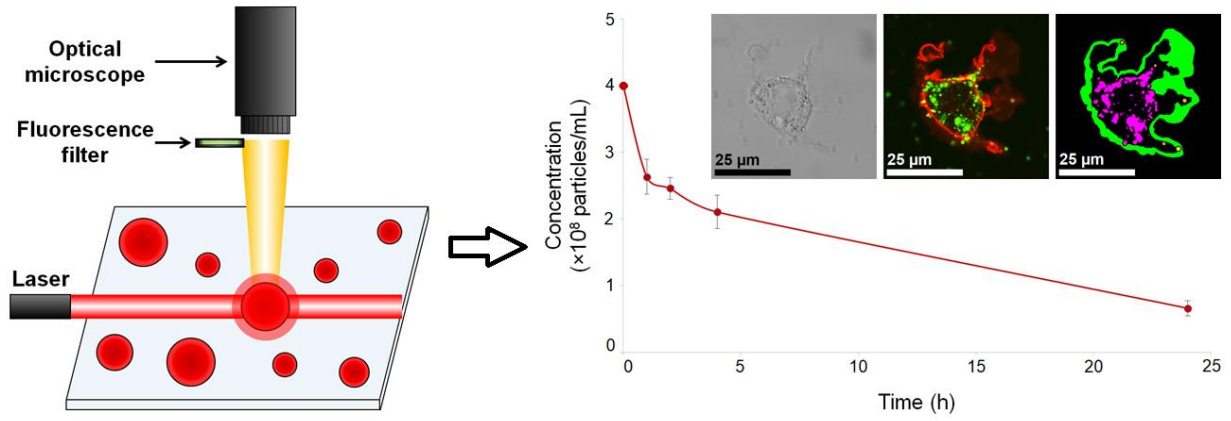












Nanoparticle tracking analysis allows a direct measurement of the amount of nanoparticles which did not interact with immune cells. Complementary techniques (such as confocal microscopy) allow to visualize nanoparticles which did interact with such cells.