

1 DOI: 10.1002/ ((please add manuscript number))

2 **Article type: Full paper**

3 **Correlative Light-Electron Microscopy shows RGD-targeted ZnO Nanoparticles**
 4 **Dissolve in the Intracellular Environment of Triple Negative Breast Cancer Cells and**
 5 **Cause Apoptosis with Intra-Tumor Heterogeneity.**

6
 7 *Basmah A. Othman*¹, *Christina Greenwood*², *Ayman F. Abuelela*³, *Anil A. Bharath*⁴, *Shu Chen*¹,
 8 *Ioannis Theodorou*¹, *Trevor Douglas*⁵, *Maskai Uchida*⁵, *Mary Ryan*¹, *Jasmeen S. Merzaban*^{3*},
 9 *Alexandra E. Porter*^{1‡}.

10

11 1. Department of Materials, Imperial College London, Royal School of Mines, Exhibition Road, London, UK
 12 SW7 2AZ.

13 2. Cell and Molecular Biology Research Laboratory, Faculty of Medical Sciences, Post Graduate Medical
 14 Institute, Anglia Ruskin University, Bishop Hall Lane, Chelmsford, UK CM1 1SQ.

15 3. Cell Migration and Signaling Laboratory, Division of Biological and Environmental Sciences and
 16 Engineering, King Abdullah University of Science and Engineering (KAUST), Thuwal, Saudi Arabia 23955-
 17 6900.

18 4. Department of Bioengineering, Imperial College London, Royal School of Mines, Exhibition Road, London,
 19 UK SW7 2AZ.

20 5. Department of Chemistry, Indiana University, 800 E. Kirkwood Avenue, Bloomington, IN, USA 47405.

21

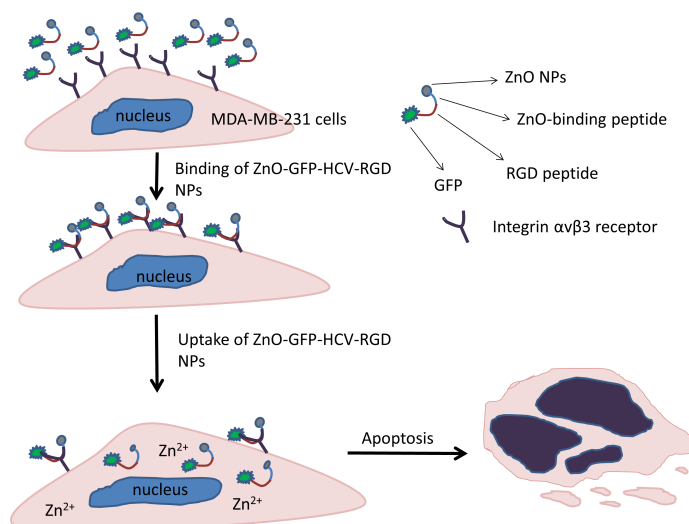
22 Address Correspondence to:

23 * Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science
 24 and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia, +966 (0)12 8082383,
 25 jasmeen.merzaban@kaust.edu.sa

26

27 ‡ Imperial College London and London Centre for Nanotechnology, London, UK, +44 (0) 207 5949691,
 28 a.porter@imperial.ac.uk

29



30

31

Schematic of binding of ZnO-GFP-HCV-RGD NPs to integrin $\alpha\text{v}\beta\text{3}$ receptors, cellular uptake, and induced apoptosis in MDA-MB-231 cells.

32

33 **Abstract** ZnO nanoparticles (NPs) are reported to show a high degree of cancer cell selectivity

34 with potential use in cancer imaging and therapy. Questions remain about the mode by which

35 the ZnO NPs cause cell death, whether they exert an intra- or extra-cellular effect, and the
36 resistance among different cancer cell types to ZnO NP exposure. The present study
37 quantified the variability between the cellular toxicity, dynamics of cellular uptake and
38 dissolution of bare and RGD (Arg-Gly-Asp)-targeted ZnO NPs by MDA-MB-231 cells.
39 Compared to bare ZnO NPs, RGD-targeting of the ZnO NPs to integrin $\alpha\beta3$ receptors
40 expressed on MDA-MB-231 cells appeared to increase the toxicity of the ZnO NPs to breast
41 cancer cells at lower doses. Confocal microscopy of live MDA-MB-231 cells confirmed
42 uptake of both classes of ZnO NPs with a commensurate rise in intracellular Zn^{2+}
43 concentration prior to cell death. The response of the cells within the population to
44 intracellular Zn^{2+} was highly heterogeneous. In addition, the results emphasize the utility of
45 dynamic and quantitative imaging in understanding cell uptake and processing of targeted
46 therapeutic ZnO NPs at the cellular level by heterogeneous cancer cell populations, which
47 could be crucial for the development of optimized treatment strategies.

48 **Keywords**; triple negative breast cancer cells, zinc oxide nanoparticles, cytotoxicity, cellular
49 targeting, integrin $\alpha\beta3$ receptors

50 **1. Introduction**

51 Breast cancer is a complex and heterogeneous disease that can be classified according to the
52 presence of predictive markers like estrogen receptor (ER), progesterone receptor (PR) and
53 human epidermal growth factor receptor 2 (HER2) [1, 2]. Over 70% of breast cancers express
54 ER and 15% overexpress HER2, therefore endocrine and anti-HER2 therapies are key
55 strategies in their management [2]. In approximately 15–20% of patients with breast cancer,
56 the tumors do not express ER or PR and do not express HER2; these are called triple-negative
57 breast cancers (TNBCs) [3]. Treatment options of TNBCs are limited because these cells do
58 not respond to hormonal therapy [2, 4]. For this reason, identification of novel, targeted
59 therapies for TNBCs would be of great benefit to patients.

60 ZnO nanomaterials (NMs) were reported to show a high degree of cancer cell
61 selectivity with potential use in cancer imaging and therapy [5-7]. Kishwar *et al.* (2010),
62 reported the use of ZnO nanowires (NWs) in cell-localized photodynamic therapy for breast
63 cancer [8]. Many studies also reported on the use of ZnO NMs as drug carriers for
64 chemotherapeutic drugs [9, 10] and in tumor imaging [11]. Zinc is an essential trace element
65 involved in a number of biological processes [12, 13]; however an increase in the local zinc
66 concentration causes cell death [14]. Zn^{2+} has many effects on cancer cells, which include
67 alteration in gene expression, reduction in cellular metabolism and induction of apoptosis [15,
68 16]. The apoptotic effects of zinc suggest that ZnO NPs can be applied as an anticancer agent
69 and provide a potential target for the development of anti-tumor agents [15]. Even more
70 striking are *in vitro* observations indicating that ZnO NPs can preferentially destroy ER
71 positive cancer cells with significantly less toxicity against normal cells [15, 17, 18].
72 However, far less information is available about whether targeted ZnO NPs can target and
73 destroy ER negative cells.

74 Debate exists around whether ZnO NMs are taken up by cancer cells and then
75 dissolve intracellularly, or whether they dissolve in the extracellular matrix releasing ionic
76 zinc which subsequently diffuses into the cells. Some studies show that ZnO NMs are
77 internalized by cancer cells and dissolve releasing a local dose of toxic Zn^{2+} ions [19]. Other
78 studies suggest that ZnO will dissolve in the lysosomes (pH~5.7) [19, 20], or in the acidic
79 microenvironment (pH~5-6) associated with cancer [16, 21], whereas they will not dissolve
80 in the microenvironment of healthy cells (pH 7.2) [16]. This is because ZnO NMs are very
81 sensitive to dissolution in acidic conditions (according to the Pourbaix diagram (or Eh-pH) of
82 ZnO [22]) *i.e.* below pH 6.7 at physiological temperature. A study by Muller *et al.* used zinc
83 sensitive dyes to show that ZnO dissolution only occurs inside macrophage cells and that
84 raised Zn^{2+} ion concentrations can be correlated directly to cell death [19]. Gilbert *et al.*

85 combined high resolution X-ray spectromicroscopy and high elemental sensitivity X-ray
86 microprobe analyses to determine the fate of ZnO and the less soluble iron-doped ZnO NPs
87 following exposure to cultures of human bronchial epithelial cells, BEAS-2B [23]. The data
88 suggested that cellular uptake of ZnO NPs is a mechanism of zinc accumulation; following
89 uptake, ZnO NPs dissolved completely generating intracellular Zn^{2+} complexed by molecular
90 ligands [24]. A combination of atomic force microscopy and scanning transmission X-ray
91 microscopy (STXM) were used [24], however being surface imaging techniques they do not
92 probe depth information and should be contrasted with techniques that do provide such
93 information. In TNBCs, direct identification of the chemical format of zinc taken up by cells
94 exposed to ZnO NPs, and its intracellular fate, has not yet been achieved.

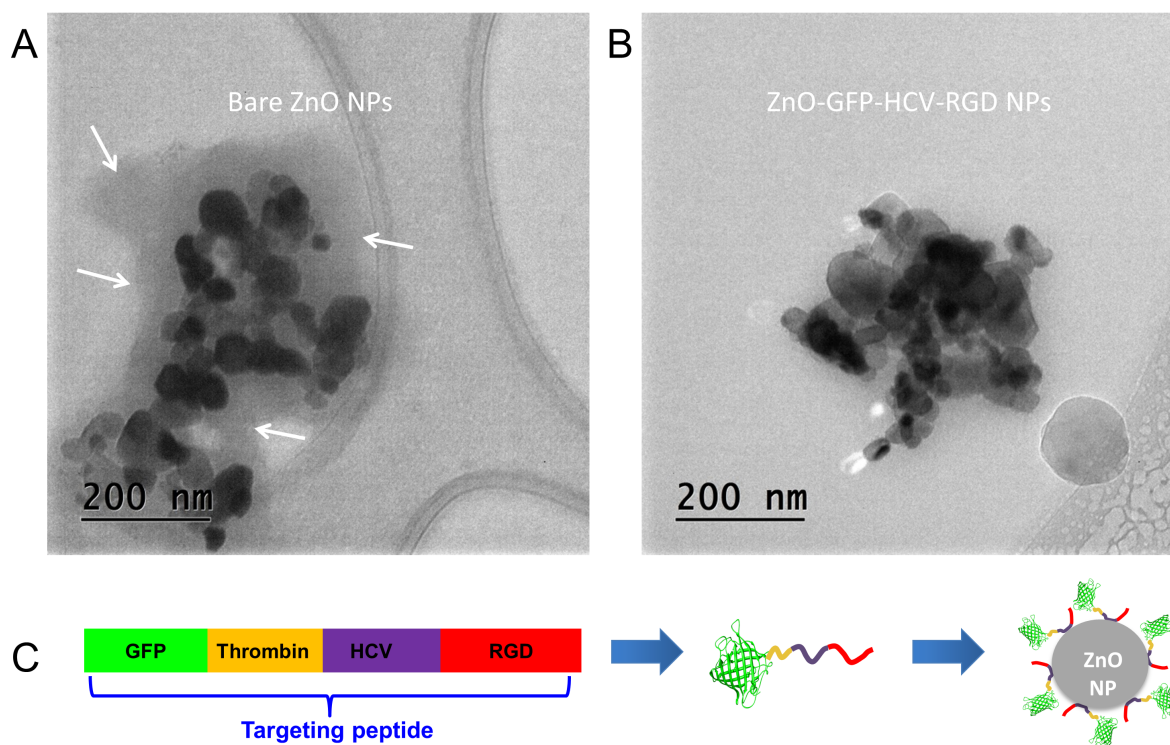
95 The aim of this study is to image the interaction of bare and ZnO-GFP-HCV-RGD
96 NPs with TNBCs *at the cellular level*, to assess whether this platform can deliver a high local
97 toxic dose of Zn^{2+} ions intracellularly to TNBCs and if there is any variability in this response
98 within the cell populations. These interactions and the potency of cell exposure to ZnO NPs
99 are compared to ER⁺ MCF-7 cells. The toxic effects of ZnO-GFP-HCV-RGD NPs on TNBCs
100 will be discussed in the context of a pH-triggered release of metal ions within the cell. It is
101 hypothesized that by targeting ZnO NPs to the integrin $\alpha\beta3$ receptors expressed in TNBCs,
102 the functionalized ZnO NPs can be targeted to cancer cells, specifically killing those cells and
103 leading to a lower therapeutic dose of ZnO NMs. Since metastatic breast tumors show a 10-
104 15 fold higher expression of $\alpha\beta3$ receptors than healthy epithelial cells, integrin $\alpha\beta3$ was
105 selected as a target [25-27] and RGD peptide was used as a targeting agent. For the first time,
106 uptake and intracellular dissolution and variability of the response of the cells within the cell
107 population of ZnO-GFP-HCV-RGD NPs by TNBCs is correlated to cell death using dynamic
108 confocal imaging.

109 **2. Results**110 **2.2. Physicochemical characterization and dissolution of ZnO**
111 **nanoparticles**

112 A range of physicochemical properties of bare and ZnO-GFP-HCV-RGD NPs were
113 characterized. Based on product information, bare ZnO NPs have a physical size ranging
114 from 10-30 nm. The dispersion state of bare and functionalized NPs in cell culture medium
115 was investigated by cryo-transmission electron microscopy (Cryo-TEM). This technique
116 allows NP solutions to be frozen rapidly and viewed directly in the frozen state, which makes
117 conventional sample drying process unnecessary, thus NPs can be analyzed close to their
118 natural state in solution, eliminating drying-induced artifacts. Cryo-TEM images show that
119 the bare and the ZnO-GFP-HCV-RGD NPs are near-spherical and form agglomerates of
120 hundreds of nanometers in MEM cell culture media containing 10 % FBS (Figure 1A and B).
121 Indeed, the hydrodynamic sizes of bare and targeted ZnO NPs in MEM with 10% FBS as
122 characterized by dynamic light scattering (DLS) are 427 ± 51.00 and 490.6 ± 14.56 nm,
123 respectively. The white arrows indicate the formation of protein corona (50-200 nm in
124 thickness) around bare ZnO NPs (Figure 1A, white arrows). However, there was no
125 pronounced protein corona formation around ZnO-GFP-HCV-RGD NPs (Figure 1B). A
126 schematic of the RGD functionalization protein attached to the ZnO NPs is shown in Figure
127 1C. The protein is composed of a cell targeting peptide domain (CDCRGDCFC), a ZnO
128 binding peptide domain (HCVAHR) and a green fluorescence protein (GFP) for tracking the
129 modified ZnO NPs by fluorescence microscopy.

130 Inductively coupled plasma-optical emission spectroscopy (ICP-OES) dissolution
131 measurements of bare ZnO NPs in different cell culture media used throughout this study, as
132 well as in HEPES confocal imaging buffer (pH=7.4) and lysosomal simulated body fluid
133 (Lyso-SBF, pH=5.2) at 37°C are presented in supplementary information, Figure S3A-B.
134 Bare ZnO NPs showed ≤ 10 $\mu\text{g/ml}$ dissolution in all cell culture media (pH 7.3) and the

135 confocal imaging buffer (pH 7.4) (Figure S3A). As expected from the Pourbaix (or Eh-pH)
 136 diagram of zinc oxide [22], bare ZnO NPs completely dissolved (450 $\mu\text{g}/\text{ml}$) in Lyso-SBF
 137 (pH=5.2) (Figure S3B). To assess the effects of the RGD-targeting peptide on the dissolution
 138 of the NPs, dissolution of the ZnO-GFP-HCV-RGD NPs (described in Supplementary
 139 information, Figure S3C) was studied in HEPES confocal imaging buffer at pH 7.4. The
 140 targeted ZnO-GFP-HCV-RGD NPs experienced a low amount of dissolution ($< 1 \mu\text{g}/\text{ml}$) in
 141 HEPES confocal imaging buffer (Figure S3C).



142

143 **Figure 1: Cryo-TEM images of (A) bare ZnO and (B) ZnO-GFP-HCV-RGD NPs.** There is pronounced formation of
 144 protein corona around bare ZnO NPs as indicated by white arrows. The spherical particle at the bottom right corner in (B)
 145 is an ice particle. (C) Simplified schematic of the functionalization peptide attached to the ZnO NPs. The peptide is composed
 146 of a targeting peptide (RGD, red) and a ZnO binding peptide (HCV, purple). To track binding of the targeting peptide to the
 147 surface of the bare ZnO NPs, the peptide was tagged with a GFP protein (green) via thrombin cleavage site (yellow).

148

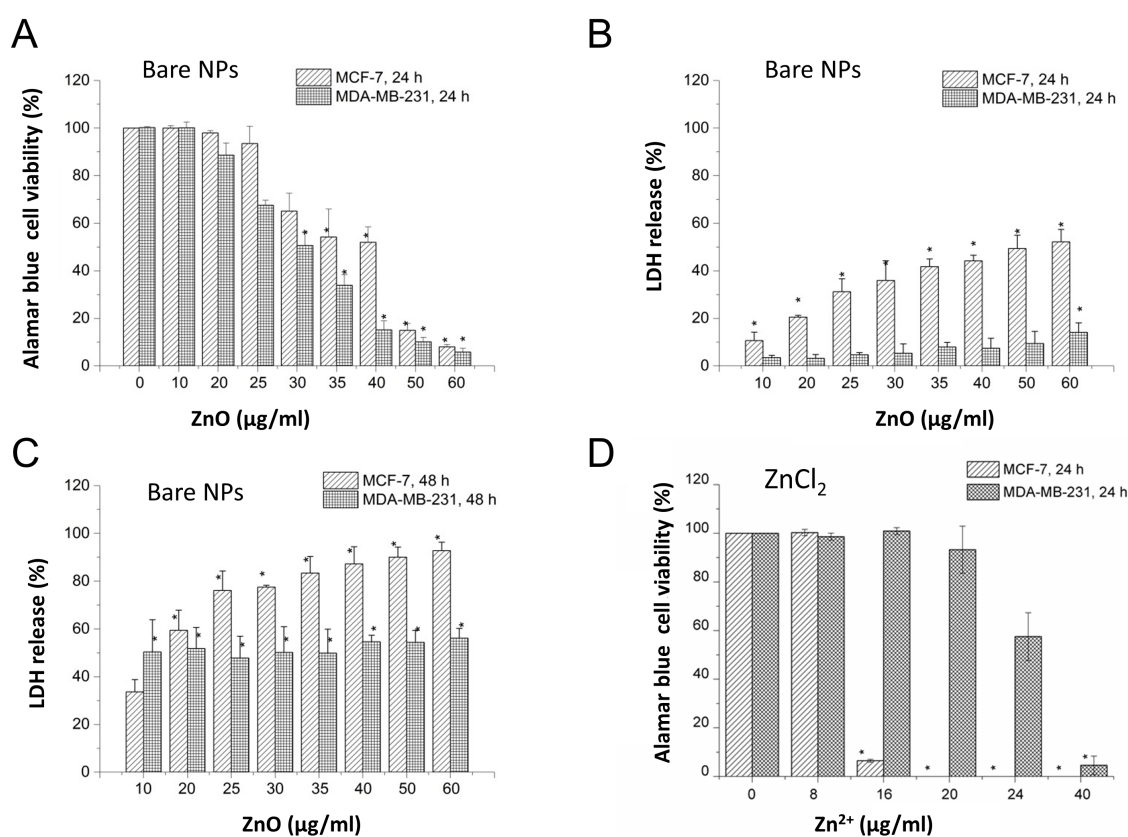
149

150 **2.3. ZnO NPs reduce the viability of MCF-7 and MDA-MB-231 breast cancer**
151 **cells**

152 In order to assess the effect of ZnO NPs on the viability of breast tumor cells, MCF-7 and
153 MDA-MB-231 cell lines were chosen as representative cell models in viability assays, as they
154 best reflect the two main subtypes of breast cancer; hormone receptor positive (ER⁺/PR⁺) and
155 hormone receptor negative, HER2 negative (TNBC) tumors, respectively [28].

156 The effects of bare ZnO NPs on the viability of both breast cancer cell lines was
157 measured using the Alamar blue (which measures the metabolic activity and viability of the
158 cells) and LDH (which measures leakage of the plasma membrane and cell death) assays
159 (Figure 2A-C). At concentrations of bare ZnO NPs between 20-60 µg/ml, the MDA-MB-231
160 and the MCF-7 cells showed a dose dependent reduction in viability as measured by the
161 Alamar blue assay (Figure 2A); compared to non-treated controls (100% viability), the
162 reduction in the viability of the cells was significant (P<0.05) at concentrations of 30-60 and
163 35-60 µg/ml in the MDA-MB-231 and the MCF-7 cells, respectively (Figure 2A). The
164 cytotoxic potency (IC₅₀) of bare ZnO NPs, reported as the 50% lethal concentration [29, 30]
165 after 24 h exposure was 30 and 36 µg/ml for the MDA-MB-231 and MCF-7 cells,
166 respectively. The LDH assay, which measures the leakage of the plasma membrane and
167 hence the cytotoxic effects of the NPs, showed that there was a significant difference
168 (p<0.05) in the LDH release between the MCF-7 and the MDA-MB-231 cells after 24 h
169 exposure to bare ZnO NPs at concentrations between 20-60 µg/ml (Figure 2B). Bare ZnO
170 NPs were more toxic to the MCF-7 cells than the MDA-MB-231 cells. Figure 2B shows that,
171 compared to the positive control (1% Triton X-100), bare ZnO NPs exhibited a significant
172 (p<0.05) dose dependent toxicity to the MCF-7 cells at all tested concentrations after 24 h
173 exposure. No clear dose dependent toxicity was measured when the MDA-MB-231 cells
174 were exposed to bare ZnO NPs at all tested concentrations; the toxicity was only significant
175 at 60 µg/ml of the bare ZnO NPs but with only <20% cytotoxic effects (Figure 2C) with the

176 LDH assay. The IC_{50} of bare ZnO NPs in the MCF-7 cells after 24 h exposure was 48 $\mu\text{g/ml}$
 177 (Figure 2B). The IC_{50} could not be determined for the MDA-MB-231 cells, since the bare
 178 ZnO NPs were only toxic to < 25% of the cells at the highest concentration tested (60 $\mu\text{g/ml}$)
 179 (Figure 2B). Since the maximum release of LDH was <20% in the MDA-MB-231 cells after
 180 24 h exposure to the bare ZnO NPs even at the highest concentration tested (60 $\mu\text{g/ml}$), a
 181 longer incubation time (48 h) was examined for both cell lines (Figure 2C). Indeed, the bare
 182 ZnO NPs induced a higher toxicity to the MCF-7 and the MDA-MB-231 cells after 48 h
 183 compared to 24 h exposure.



184

185 **Figure 2: Exposure to bare ZnO NPs reduces viability of MCF-7 and MDA-MB-231 cells.** (A) Alamar blue viability
 186 assay of MCF-7 and MDA-MB-231 breast cancer cells exposed to bare ZnO NPs for 24 h. (B and C) LDH cytotoxicity
 187 assay of MCF-7 and MDA-MB-231 breast cancer cells exposed to bare ZnO NPs for 24 h and 48 h, respectively. (D) Alamar
 188 Blue viability assays of MCF-7 and MDA-MB-231 cells exposed to Zn^{2+} (0, 8, 16, 20, 24 and 40 $\mu\text{g/ml}$, equivalent to zinc
 189 ions released from 0, 10, 20, 25, 30 and 50 $\mu\text{g/ml}$ of ZnO) for 24 h. Cells treated under similar conditions but without ZnO
 190 NPs or ZnCl_2 were used as negative controls for the Alamar Blue assay and cells treated with Triton X-100 were used as a
 191 positive control for the LDH assay. The values represent the mean \pm STD of three experiments each performed in triplicate.
 192 * $p \leq 0.05$.

193 To evaluate whether the toxic effects of the ZnO NPs were related to a high local dose
194 of Zn^{2+} ions in the extracellular milieu or an effect of an elevated local dose of Zn^{2+} , the
195 MCF-7 and MDA-MB-231 breast cancer cells were exposed to ionic zinc (Figure 2D).
196 Neither breast cancer cell lines experienced a reduction in their viability after exposure to
197 small doses of ionic zinc $\leq 8 \mu\text{g/ml}$ for 24 h. At higher concentrations of ionic zinc (≥ 16
198 $\mu\text{g/ml}$), each cell line experienced a different response; whereas the MCF-7 cells showed a
199 sudden loss in viability at concentrations $\geq 16 \mu\text{g/ml}$, only a 40% reduction in the viability of
200 the MDA-MB-231 cells was observed at concentrations as high as $24 \mu\text{g/ml}$, and a significant
201 loss of the viability ($>90\%$) was observed only at $40 \mu\text{g/ml}$ of ionic zinc (Figure 2D). Taken
202 together, these results indicate a difference between the cellular interactions of ionic zinc and
203 particulate ZnO NPs.

204 The effect of bare ZnO NPs on the percentage of apoptotic and necrotic population of
205 the breast cancer cells was investigated using flow cytometric analysis of Annexin V/7AAD
206 stained cells (Supplementary information, Figure S4). Overall, exposure of MDA-MB-231
207 cells to bare ZnO NPs leads to apoptosis while exposure to MCF-7 cells leads to necrosis
208 (Supplementary information, Figure S4).

209 It was hypothesized that RGD-targeted ZnO NPs may selectively target and destroy
210 the MDA-MB-231 TNBCs and MCF-7 (ER^+ , HER2^-) at lower doses compared to bare ZnO
211 NPs. The expression of the integrin $\alpha\beta 3$ receptors in the MCF-7 and MDA-MB-231 cells
212 was investigated (Supplementary information, Figure S5). MDA-MB-231 cells showed an
213 increased expression of the integrin $\alpha\beta 3$ receptors compared to the MCF-7 cells
214 (Supplementary information, Figure S5). The effect of the targeted ZnO NPs on the viability
215 of both breast cancer cell lines was measured (Supplementary information, Figure S6). The
216 ZnO-GFP-HCV-RGD NPs were toxic to the MCF-7 and MDA-MB-231 cells with an IC_{50} of
217 $16 \mu\text{g/ml}$ and $22 \mu\text{g/ml}$ after 24 h exposure, respectively (Supplementary information, Figure

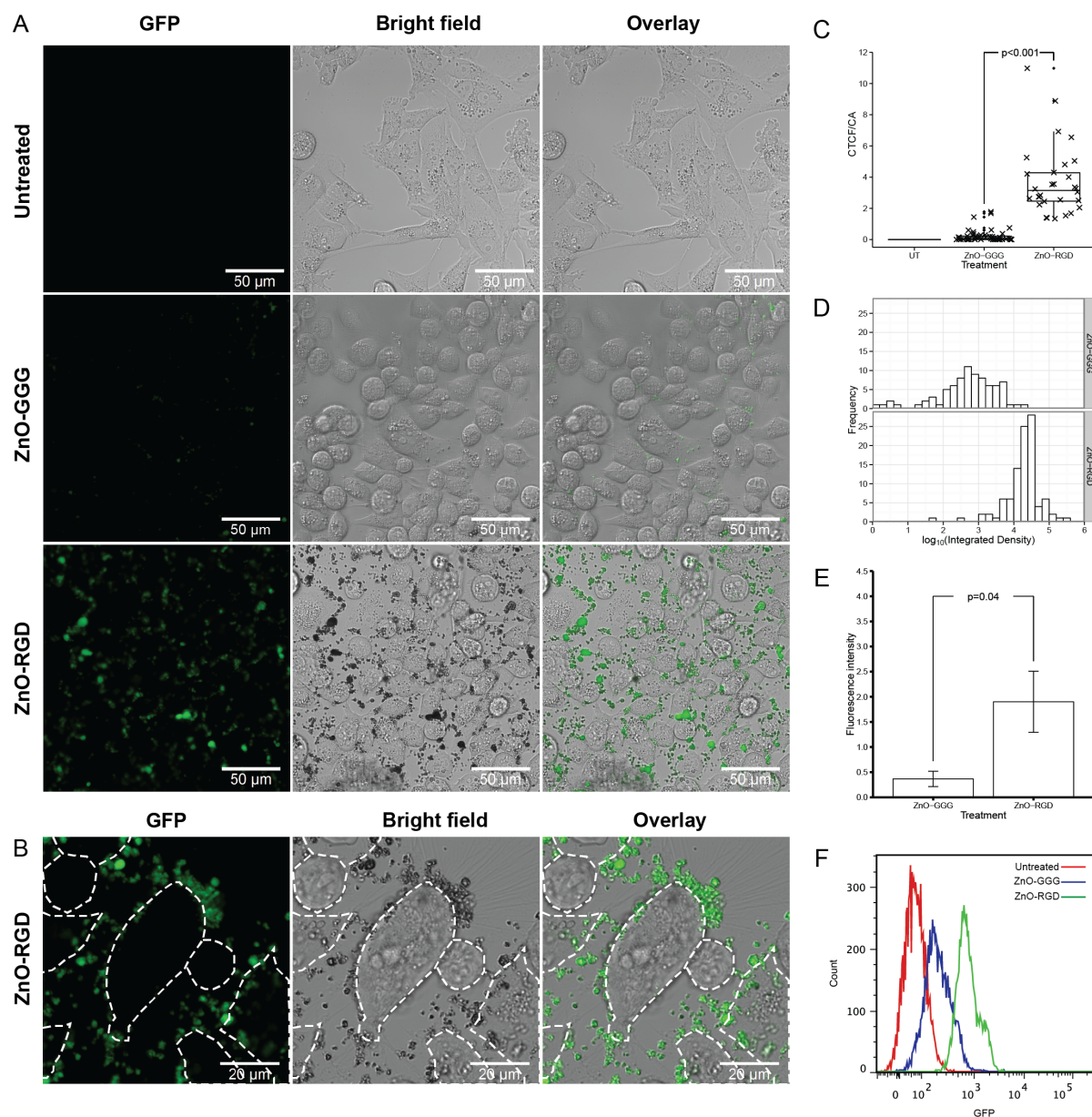
218 S6). The MCF-7 and MDA-MB-231 cells showed a significant drop in their viability
219 ($p < 0.05$) to $< 20\%$ at 20 and 25 $\mu\text{g/ml}$ of the ZnO-GFP-HCV-RGD NPs, respectively
220 (Supplementary information, Figure S6). The higher sensitivity expressed by the MCF-7 cells
221 to the RGD-targeted ZnO NPs, despite their lower expression of the integrin receptors, could
222 be attributed to the higher sensitivity of the cells to the effects of ionic zinc (refer to Figure
223 2D).

224 **2.4. The targeted ZnO-HCV-RGD-GFP NPs bind to and are taken up by** 225 **TNBCs**

226 Treatment of TNBCs is limited and challenging due to a lack of targeted therapy, aggressive
227 behavior and relatively poor prognosis, therefore, using MDA-MB-231 cells as a model, the
228 uptake and dissolution of the ZnO NPs was of key focus in our studies in order to identify
229 novel, targeted therapies for patients. MDA-MB-231 cells showed an increased expression of
230 the integrin $\alpha\beta 3$ receptors compared to the MCF-7 cells (Supplementary information, Figure
231 S5), which makes them a prime target for the ZnO-GFP-HCV-RGD NPs. Indeed, MDA-MB-
232 231 cells bound ZnO-GFP-HCV-RGD NPs more specifically over the control, ZnO-GFP-
233 HCV-GGG NPs (Figure 3).

234 The preferential ability of RGD-conjugated ZnO-NP to bind MDA-MB-231 cells was
235 determined by various assays. In order to assess the targeting capability of RGD-conjugated
236 NPs, MDA-MB-231 cells were either treated with RGD- or GGG-conjugated ZnO NPs for 30
237 minutes. A dose as high as 150 $\mu\text{g/ml}$ of the ZnO NPs was used to follow the binding of the
238 NPs within a reasonable time frame. Figure 3A shows a higher binding capacity of RGD-
239 conjugated particles compared to GGG-conjugated particles using laser scanning confocal
240 microscopy. Figure 3B shows that the RGD-conjugated NPs tend to form aggregates and bind
241 to the surface of the cells. A closer look at the boundaries of the cells (Figure 3B) shows that
242 the distribution of the particles is heterogeneous around each cell, possibly due to
243 heterogeneity in $\alpha\beta 3$ expression across the surface of the cell. With the aim of quantifying

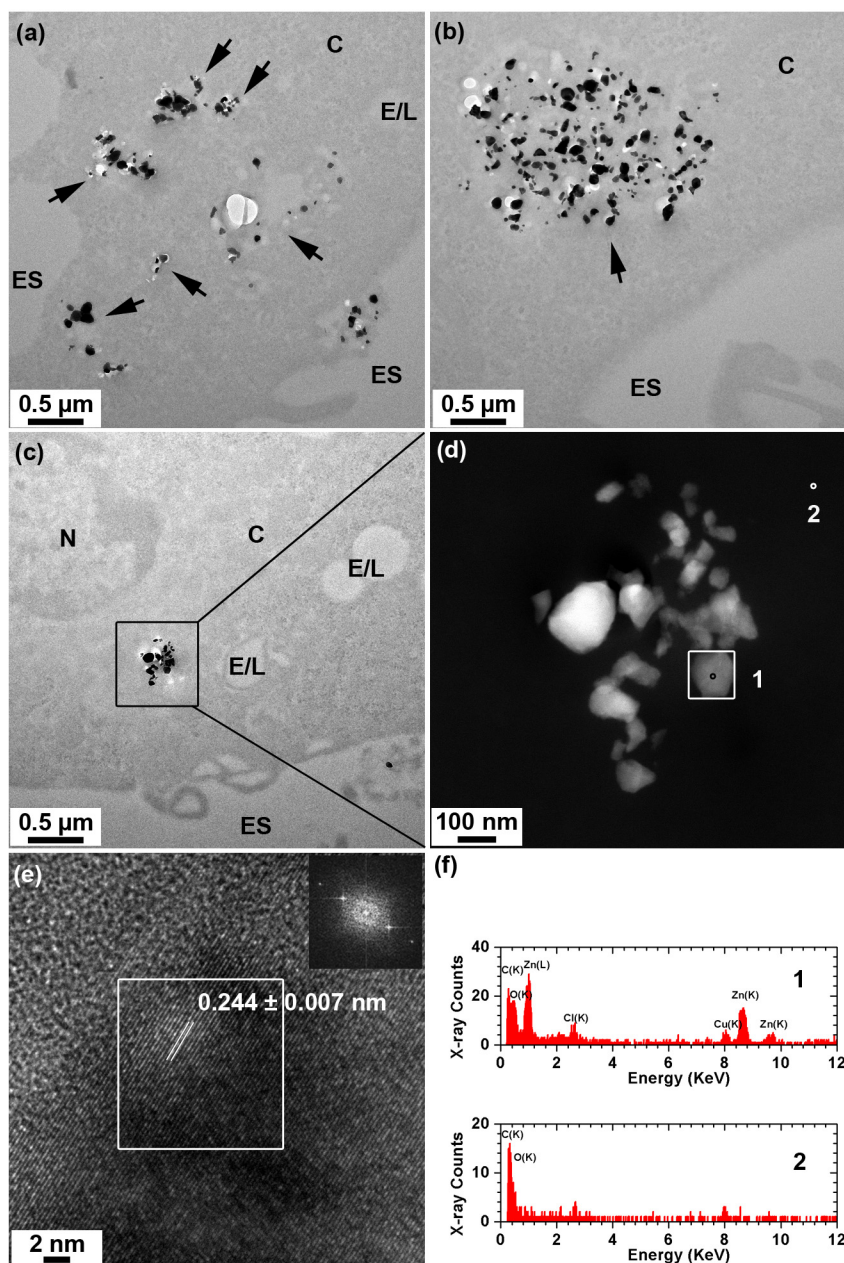
244 the difference in binding capacity between RGD- and GGG-conjugated NPs, we measured
245 the fluorescence intensity of the area representing a cell and its bound NPs (Figure 3C). Total
246 cell fluorescence (TCF) was measured and the integrated density in the area of one cell and
247 its bound NPs and was corrected to the signal from the background to yield the corrected total
248 cell fluorescence (CTCF). In order to preclude a cell size bias in our calculations, CTCF was
249 divided by cell area (CA). CTCF/CA mean for RGD-treated cells ($3.7 \pm \text{STD. deviation} = 2.2$)
250 was 16 times higher than that of GGG-treated cells ($0.22 \pm \text{STD. deviation} = 0.4$) with a 95%
251 confidence interval of 3.45 ± 1.7 ($p < 0.001$). In order to avoid the cell selection bias, integrated
252 density values were measured for 100 equally-sized tiles from RGD and GGG conditions
253 (Figure 3D). A histogram shows the difference in the distribution of the \log_{10} value of
254 integrated densities. Integrated densities were 18.7 higher in RGD condition
255 (mean = $31391 \pm \text{STD. deviation} = 43793$) compared to GGG (mean = $1675 \pm \text{STD.}$
256 deviation = 2724) with a 95% confidence interval of 29716 ± 8709 ($p < 0.001$), signifying the
257 greater ability of RGD-conjugated particles to bind MDA-MB-231 cells. Using a
258 fluorometry-based assay, Figure 3E shows the difference in binding capacity of ZnO-GFP-
259 HCV-RGD over ZnO-GFP-HCV-GGG NPs to MDA-MB-231 cells from three independent
260 experiments. The mean fluorescence intensity (MFI; $1.90 \pm \text{STD. deviation} = 0.61$) for ZnO-
261 GFP-HCV-RGD-treated cells was 5.18 times higher than that of ZnO-GFP-HCV-GGG-
262 treated cells ($0.37 \pm \text{std. deviation} = 0.15$) with a 95% confidence interval of 1.52 ± 1.39
263 ($p < 0.05$). Finally, flow cytometric analysis (Figure 3F) also revealed that the MFI is over a 3-
264 fold higher for cells treated with ZnO-GFP-HCV-RGD NPs over the ZnO-GFP-HCV-GGG
265 control. Overall, these studies unequivocally show the preferential binding of ZnO-GFP-
266 HCV-RGD NPs over ZnO-GFP-HCV-GGG NPs to MDA-MB-231 cells.



267
 268 **Figure 3: RGD-mediated binding of ZnO-GFP-HCV-RGD NPs to MDA-MB-231 cells.** (A) Confocal images of MDA-
 269 MB-231 cells either left untreated (UT) or treated with 150 $\mu\text{g}/\text{mL}$ of ZnO-GFP-HCV-GGG or ZnO-GFP-HCV-RGD NPs
 270 for 30 min. The figure shows higher binding capabilities of RGD-coupled NPs compared to GGG-coupled NPs. The figures
 271 are representative of $n=3$ independent experiments. (B) Higher magnification confocal images of ZnO-GFP-HCV-RGD-
 272 treated cells showing direct binding of ZnO-GFP-HCV-RGD NPs (green) to the surface of MDA-MB-231 cells. Dotted lines
 273 represent the boundaries of the cells. (C) Box plot of fluorescence intensity observed in confocal experiments represented in
 274 (A) denoted as CTCF/CA (see Methods). Raw values are represented by individual crosses (x). (D) Representative confocal
 275 images were divided into 100 tiles and integrated density was calculated. A histogram of \log_{10} (integrated density) shows a
 276 positive shift in case of ZnO-GFP-HCV-RGD compared to ZnO-GFP-HCV-GGG. (E) Fluorescence spectroscopy analysis
 277 of MDA-MB-231 cells that were either left untreated or treated with ZnO-GFP-HCV-GGG or ZnO-GFP-HCV-RGD
 278 particles. The fluorescence signal from untreated cells was used as a blank that was subtracted from the ZnO-GFP-HCV-
 279 RGD and ZnO-GFP-HCV-GGG samples. Error bars represent the standard deviation of $n=3$ independent experiments. (F)
 280 Representative flow cytometric histograms showing the green fluorescence intensity of MDA-MB-231 cells either left
 281 untreated or following treatment with ZnO-GFP-HCV-GGG or ZnO-GFP-HCV-RGD NPs. This is a representative
 282 histogram of $n=3$ independent experiments. Statistical significance of differences was determined by two-sided unpaired
 283 student's t test and significance was defined as $p < 0.05$. For all binding experiments performed here, NPs were incubated with
 284 the MDA-MB-231 cells in binding buffer containing CaCl_2 at 4°C .

285 In order to show that, after binding to MDA-MB-231 cells, the ZnO-GFP-HCV-RGD NPs
286 were internalised by, and dissolved inside the TNBCs, TEM images were taken to show the
287 localisation and morphology of the ZnO-GFP-HCV-RGD NPs inside the cells after 3 h
288 exposure (Figure 4). None of the conventional heavy metal staining processes, like osmium
289 staining, was applied during TEM sample preparation. This compromises the contrast of cell
290 ultrastructure, however, minimises the artificial alteration of the chemistry of particles [31].
291 Low resolution bright field TEM (BF-TEM) images (Figure 4A-C) show that the particles are
292 internalised by the cells and are found as individual particles (Figure 4A), as well as clusters
293 of particles in endosome/lysosome-like vesicles and the cytoplasm (Figure 4A and B). High
294 angle annular dark field scanning transmission electron microscopy (HAADF-STEM)
295 imaging is a Z (atomic number)-contrast imaging technique, therefore this method shows
296 local variations in the atomic number within a sample. HAADF-STEM images (Figure 4D)
297 highlight the morphology of ZnO NPs present in the cytoplasm from the boxed area in Figure
298 4C, which had partially dissolved. A representative high resolution TEM (HRTEM) image
299 (Figure 4E) of the ZnO NPs labelled by a box in Figure 4D shows crystalline lattice fringes
300 with an interplanar spacing consistent with bulk ZnO (011) lattice planes (Inorganic Crystal
301 Structure Database, Ref.ICSD82028) (Figure 4E). A STEM-EDX spectrum taken from the
302 particle (area 1 in (D)) further confirms the presence of Zn; the peaks at around 1.0, 8.6 and
303 9.6 keV correspond to Zn(L) and Zn(K) peaks, respectively (Figure 4F, *plot 1*); while the
304 STEM-EDX spectrum taken from the background (area 2 in (D)) does not show any trace
305 of Zn (Figure 4F, *plot 2*).

306



307

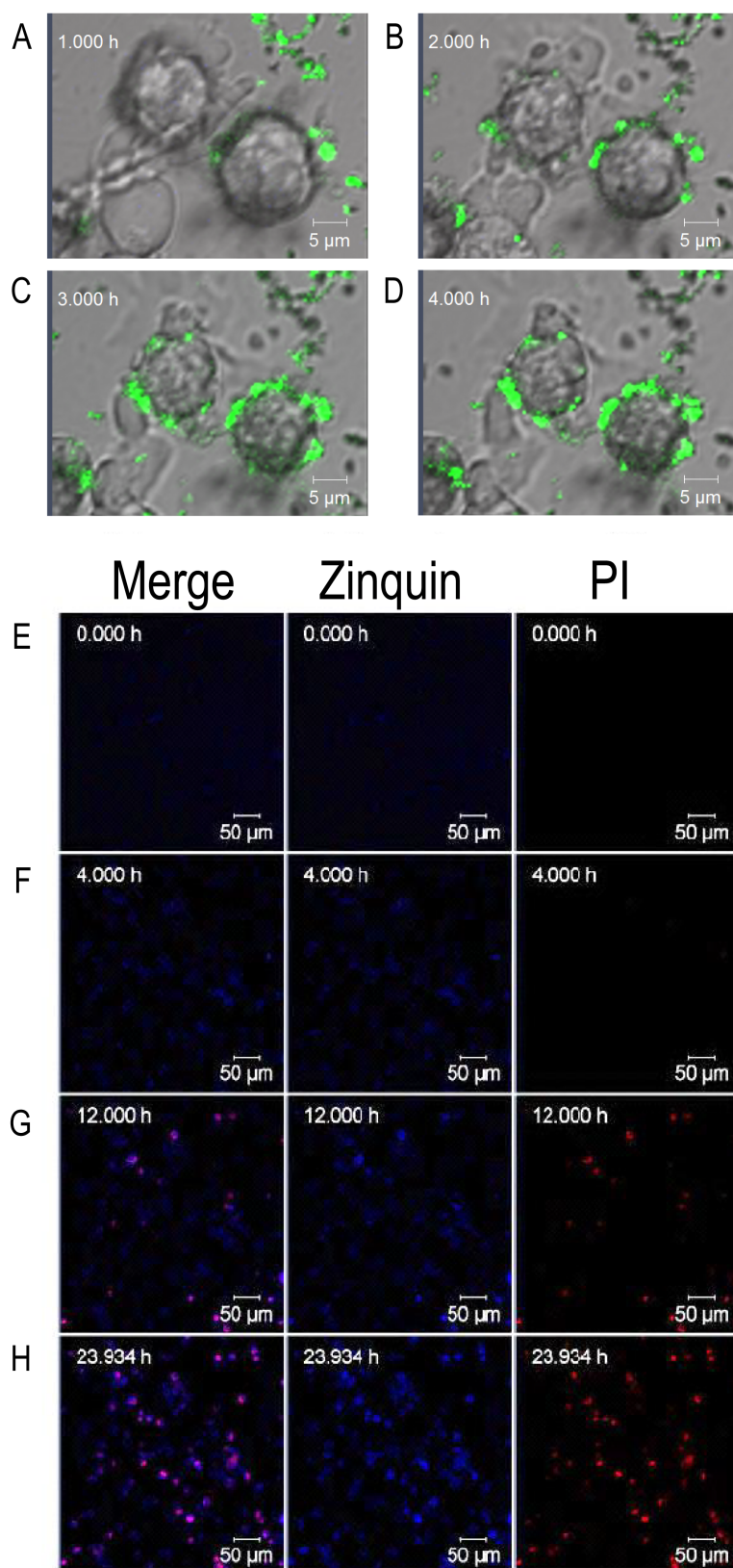
308 **Figure 4: TEM analysis of MDA-MB-231 cells exposed to 30 $\mu\text{g}/\text{ml}$ ZnO-GFP-HCV-RGD NPs for 3 h. (A-C) BF-TEM images**
 309 **show the presence of ZnO NPs in endosome/lysosome-like structures (marked by arrows) and the cytoplasm. (D) A HAADF-**
 310 **STEM image highlights the morphology of ZnO NPs, taken from the boxed region in (C). (E) HRTEM shows the crystalline**
 311 **lattice fringes of a NP from the boxed area in (D), the interplanar spacing is consistent with bulk ZnO (011) lattice planes**
 312 **(Inorganic Crystal Structure Database, Ref.ICSD82028). The inset is the FFT pattern taken from the boxed area. (F) STEM-**
 313 **EDX spectra (1 and 2) taken from the particle and the background (area 1 and area 2 in (D), respectively). The peaks at**
 314 **around 1.0, 8.6 and 9.6 keV in spectrum 1 correspond to Zn(L) and Zn(K) peaks, respectively. ES = extracellular space, C =**
 315 **cytoplasm, E/L = endosome/lysosome and N = nucleus.**

316 **2.5. Rise of intracellular Zn^{2+} precedes cell death.**

317 **Targeted ZnO NPs.** To correlate binding of the ZnO-GFP-HCV-RGD NPs to cells and
 318 intracellular ionic Zn^{2+} release to cell death, we applied dynamic confocal microscopy. For

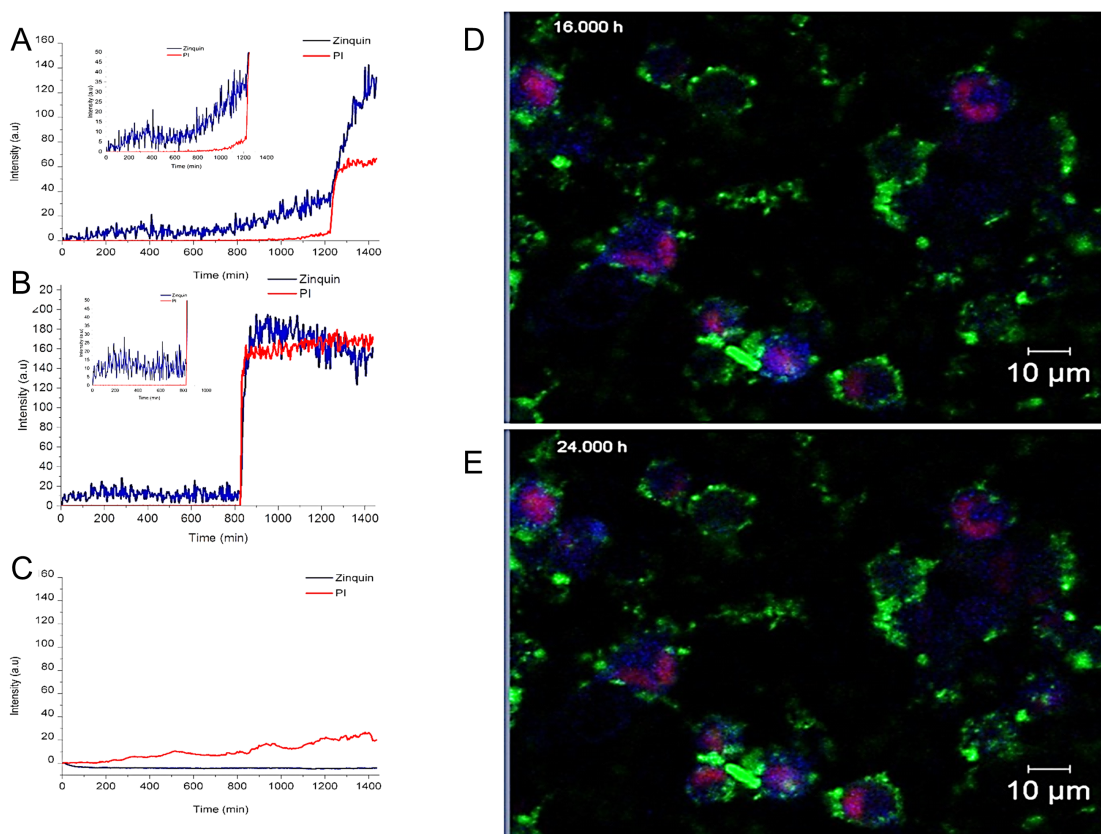
319 these experiments three different dyes were used to track the NPs, Zn^{2+} release and cell death,
320 and the heterogeneity of the cell response was quantified (A video of intracellular dissolution
321 of the targeted ZnO-GFP-HCV-RGD NPs by live MDA-MB-231 cells is in Supplementary
322 information; Video 1). To follow the rise of intracellular zinc within a reasonable time frame,
323 a dose as high as 150 $\mu\text{g/ml}$ of the ZnO NPs was used [32]. Figure 5A-D shows binding of
324 the ZnO-GFP-HCV-RGD NPs (green) to the cell membrane of the MDA-MB-231 cells over
325 time. After 1 h exposure, the cells were surrounded by a small number of the ZnO-GFP-
326 HCV-RGD NPs aggregates (Figure 5A). Over time, significantly more ZnO NPs bound to the
327 cells (Figure 5B and 4C), forming a layer of ZnO-GFP-HCV-RGD NPs surrounding the cells
328 after 4 h of exposure (Figure 5D). Again, as indicated earlier in Figure 3B, binding of the
329 ZnO-GFP-HCV-RGD NPs to the cell membrane of the MDA-MB-231 cells was
330 heterogeneous (Figure 5D). After binding to the surface of the MDA-MB-231 cells, the ZnO-
331 GFP-HCV-RGD NPs dissolved releasing ionic zinc (Zn^{2+}) (Figure 5E-H). In some cells, this
332 release of ionic zinc (Zn^{2+}) was accompanied by the increase in the intensity of Zinquin
333 (blue) inside the cells, which was clear after 4 h of exposure (Figure 5F) and the rise in the
334 intracellular zinc correlated to cell death, as indicated by the penetration of PI (red) into the
335 cells and the rise of its intracellular concentration (Figure 5G-H). However, this process was
336 variable within the cell population and in some cells PI release was not preceded by Zinquin
337 release, or occurred at the same time as Zinquin release. Analysis of the cells after exposure
338 to the targeted ZnO-GFP-HCV-RGD showed that 20% of the cells in the imaging field died
339 as early as 6 h exposure and almost 80% of the cells died after 12 h exposure. Analysis of the
340 confocal data shown in Figure 5 was used to generate representative intensity curves of
341 Zinquin and PI (Figure 6A-B). The intensity curves of Zinquin and PI from the cells exposed
342 to the RGD-targeted ZnO NPs show a gradual increase in Zinquin intensity over time and a
343 sharp increase in PI once a significant concentration of Zn^{2+} is reached (Figure 6A and B). In

344 contrast, the untreated control MDA-MB-231 cells, imaged under the same conditions, did
345 not show any increase in intracellular zinc throughout the confocal imaging experiments
346 confirming that the Zn^{2+} indicator did not fluoresce over time, in the absence of zinc (Figure
347 6C). Figure 6D-E shows representative confocal images of the dissolution of ZnO-GFP-
348 HCV-RGD ZnO NPs inside MDA-MB-231 cells after 16 and 24 h, respectively
349 (Supplementary information, video 1).



350

351 **Figure 5: (A-D) Bright field and confocal images of MDA-MB-231 cells exposed to 150 µg/ml ZnO-GFP-HCV-RGD**
 352 **NPs (green) showing binding of the NPs to the surface of the cell after (A) 1 h, (B) 2 h, (C) 3 h, and (D) 4 h exposure.**
 353 **(E-H) Confocal images showing MDA-MB-231 cells exposed to 150 µg/ml of the ZnO-GFP-HCV-RGD NPs led to the**
 354 **release of Zn²⁺ inside the cells over time; (E) 0 h, (F) 4 h, (G) 12 h, and (H) 24 h, as indicated by the increase in intensity**
 355 **of the Zn²⁺ indicator, Zinquin (blue). Toxic levels of Zn²⁺ ions inside the cell subsequently resulted in cellular penetration of**
 356 **PI (red) indicating the loss in viability and membrane integrity.**



357

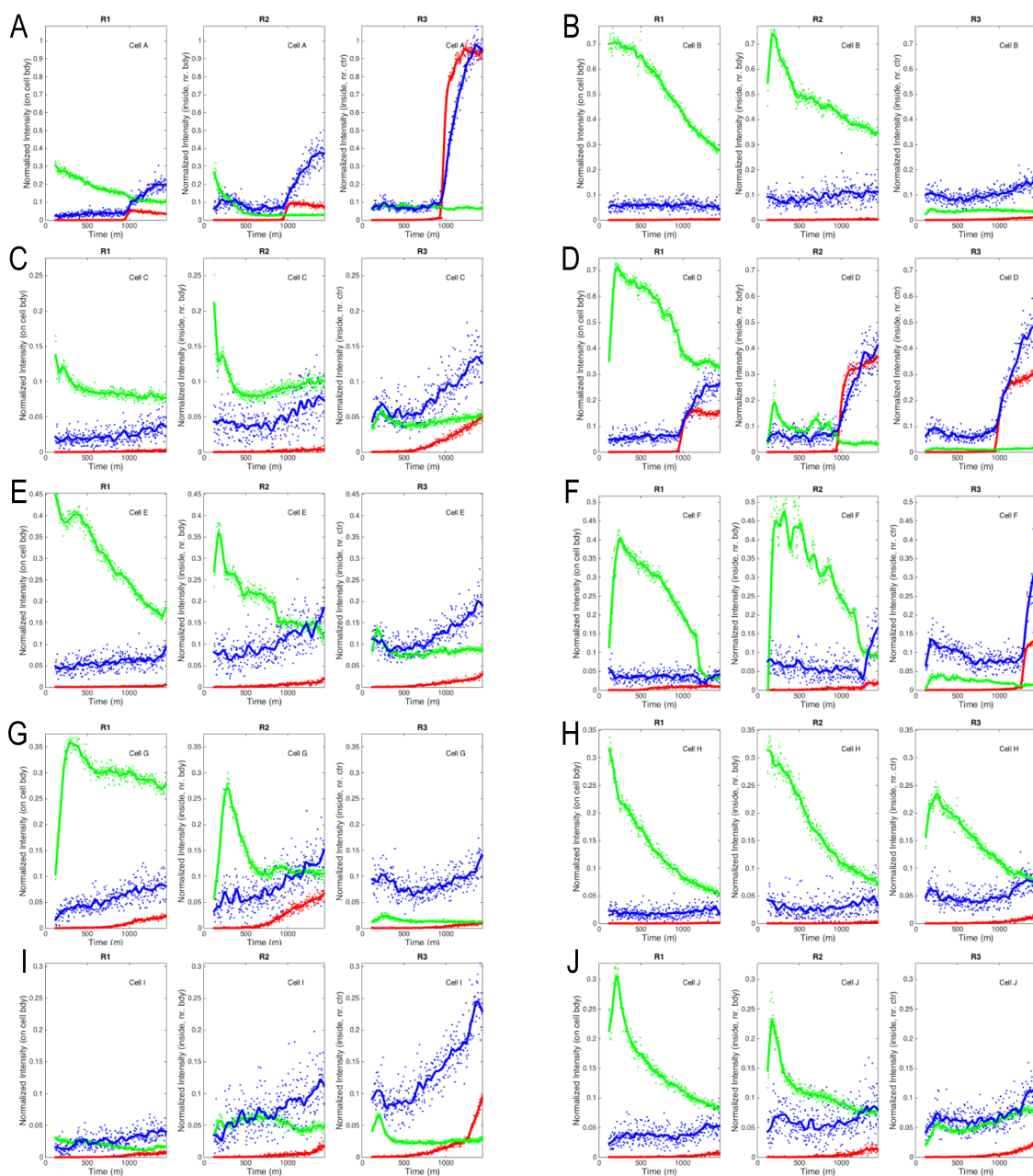
358 **Figure 6: (A-C) Intensity curves of Zinquin and PI in MDA-MB-231 cells after exposure to ZnO-GFP-HCV-RGD**
 359 **NPs showing an increase in intracellular Zn²⁺ and subsequent cell death.** The MDA-MB-231 cells were loaded with the
 360 cell-permeant Zn²⁺ indicator Zinquin-AM prior to incubation with (A and B) 150 μg/ml the targeted NPs, (C) imaging buffer
 361 (NA control). The insets in A and B show a gradual increase in Zinquin intensity over time. Fluorescent intensities were
 362 measured within a representative region of interest (ROI). **(D and E)** Confocal images showing dissolution of ZnO-GFP-
 363 HCV-RGD NPs inside MDA-MB-231 cells after 16 and 24 h, respectively. The ZnO-GFP-HCV-RGD NPs released Zn²⁺
 364 inside the cells, which was accompanied by the increase in the intensity of Zinquin (blue). Toxic levels of Zn²⁺ ions inside
 365 the cell resulted cellular penetration of PI which stained the nuclei of dead cells red. Zinquin fluoresces blue upon binding
 366 ionic zinc (Zn²⁺). Images were acquired over a time course of 24 h.

367 Cellular heterogeneity is a crucial aspect of population characteristics especially in
 368 cancerous cells, which dictates treatment response and resistance issues associated with such
 369 heterogeneity [33]. To detect cellular variability in response to ZnO NPs, measurements were
 370 made on 10 individual cells using a region tracking algorithm to “follow” the Zinquin and PI
 371 intensities at different regions of the cells. Three locations were selected (these are described
 372 in Supplementary information, Figure S7) to act as the centers for sampling color channel
 373 intensity: a) on the boundary of cells (R1), b) 75% of the distance between the cell’s center
 374 and boundary location (R2), c) within the center of a cell (R3). This sampling was used to

375 construct weighted intensity samples from the three regions sampled within each cell. Figure
376 7 illustrates that the cellular response to the ZnO-GFP-HCV-RGD NPs is highly
377 heterogeneous within the cell population. In fact, Figure 7B, D, E, G, and J showed a lag over
378 time in the position of the ZnO-GFP-HCV-RGD signal (green) at R1 and R2, indicating that
379 the particles are entering the cells. For some cells, there is a slow increase in Zinquin
380 followed by a rapid increase in PI intensities (e.g. Figure 7A and 7D). For most of the other
381 cells, the rate of increase of PI and Zinquin intensities inside the cells is less rapid (Figure
382 7B-C, and 7E-J). There were regular oscillations in the Zinquin and PI intensities inside the
383 cells; it has been reported that Zn^{2+} dynamics may modulate Ca^{2+} signaling [34], and since
384 the PI fluorescence itself relies directly on the amount of Ca^{2+} in the growth solution [35],
385 oscillations in the PI and Zinquin signaling could occur. The GFP signal was constant in R3
386 over time, which could indicate dissolution of the ZnO-GFP-HCV-RGD NPs (Figure 7A-J).

387 The absolute value of correlation coefficient ($|p|$) between the red and blue
388 illumination signal levels from regions inside and outside of cells showed a highly significant
389 correlation (KS statistic of 0.97) between ionic zinc and PI within cells with no discernible
390 difference in the distributions (KS statistic of 0.03) in regions outside the cells (Figure 8A
391 and 8B, respectively).

392

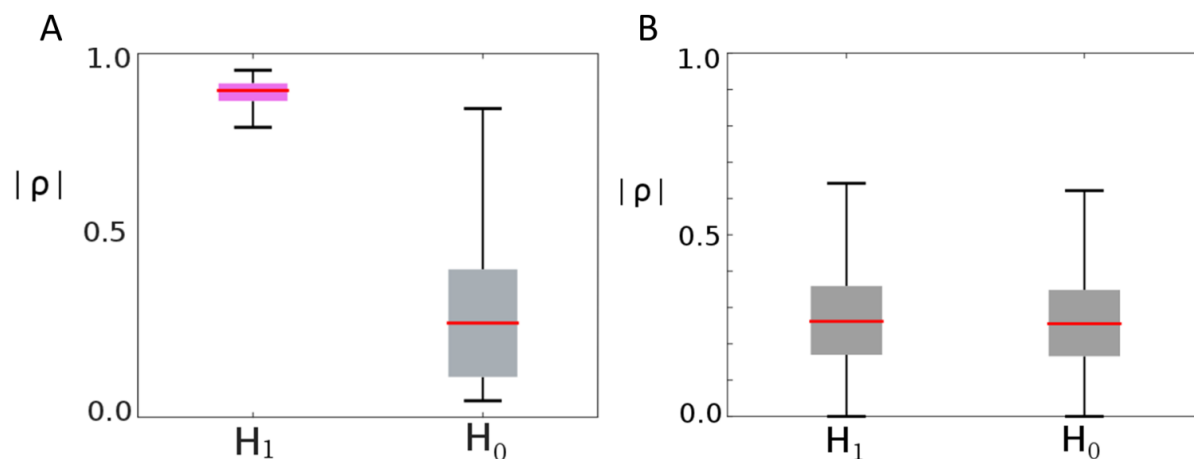


393

394 **Figure 7: Analysis of the variability of the responses of the MDA-MB-231 cells to the ZnO-GFP-HCV-RGD ZnO NPs**
 395 **as a function of time showing the color channel signals after normalization (Red = PI, Blue = Zinquin, Green = ZnO-**
 396 **GFP-HCV-RGD NPs). The red, blue and green signal channels were extracted from 10 different cells (A-J) at three different**
 397 **locations within the cell: R1 is on the boundary of cells, R2 is 75% of the distance between the cell's center and boundary**
 398 **location, and R3 is within the center of a cell. The raw data are illustrated with points, and the smoothed curves for each**
 399 **channel are also shown (Sovitzkay-Golay smoothing).**

400

401



402

403 **Figure 8: Box and whisker plots [36] of the absolute values of the correlation coefficient ($|\rho|$) between Zinquin (zinc-**
 404 **sensitive dye) levels and PI observed (A) within and (B) outside MDA-MB-231 TNBCs.** Each panel shows a comparison
 405 of correlation values in sampled regions (H_1) compared against the null hypothesis (H_0). **(A)** Regions inside the cells showed
 406 highly significant correlation (KS Statistic of 0.97) between Zinquin and PI. **(B)** Regions outside the cells showed no
 407 discernible difference in the distributions (KS Statistic of 0.03). Red lines indicate the median values, shaded areas indicate
 408 region between 25th and 75th percentiles, and whiskers indicate extreme values. See **Methods** for details on how samples
 409 were drawn from the null hypothesis.

410 **Bare ZnO NPs.** The kinetics of intracellular release of ionic Zn^{2+} and cell death were further
 411 investigated for the bare ZnO NPs using confocal laser microscopy of live breast cancer cells.
 412 These processes were also repeated after the exposure of the cells to ionic zinc from $ZnCl_2$.
 413 Figure 9 shows the intensity curves of the green fluorescent zinc-sensitive dye (Fluozin3) and
 414 PI (red), as well as the confocal images of MDA-MB-231 cells after exposure to the bare NPs
 415 and $ZnCl_2$. To follow the rise of intracellular Zn^{2+} within a reasonable time frame, a dose as
 416 high as 150 $\mu\text{g/ml}$ of the bare ZnO NPs was used [32]. In order to measure the amount of
 417 intracellular ionic zinc released and cell membrane integrity of the MDA-MB-231 exposed to
 418 the bare ZnO NPs, the fluorescent intensities of the Zn^{2+} -sensitive dye (FluoZin3) and PI
 419 were measured for 17 cells in the imaging frame over 16 h exposure. The intensities of the
 420 regions of interest (ROI) selected from the cells could not be averaged as the cells showed a
 421 heterogeneous response to the particles. Representative ROI are shown in Figure 9A and B.
 422 After exposure to the bare ZnO NPs, the MDA-MB-231 kinetics of Zn^{2+} ion release and
 423 resistance to cell death between the cells in the field of view was markedly different. 41% of
 424 the MDA-MB-231 cells (7/ 17) required 1-1.5 h to reach the maximum concentration of ionic

425 zinc (Zn^{2+}) in the cytoplasm. In contrast, 59% of cells (10/17) required 3-4 h to reach the
426 highest levels of intracellular Zn^{2+} . Once the concentration of Zn^{2+} had reached a maximum
427 inside the cell, some cells died immediately (Figure 9A). This correlation was monitored by
428 increasing intracellular levels of PI and a drop in FluoZin3 dye (Figure 9A), indicating a loss
429 of plasma membrane integrity. Figure 9B demonstrate other cells that survived for longer
430 time at the highest levels of Zn^{2+} (plateau of FluoZin3).

431 The rise of intracellular levels of Zn^{2+} and its effect on the cell viability in the MDA-
432 MB-231 cells after exposure to ZnCl_2 were subsequently compared to those of the bare ZnO
433 NPs. ZnCl_2 was used at a concentration of 250 $\mu\text{g/ml}$. This concentration of ZnCl_2 was
434 chosen as it has the same Zn^{2+} concentration of 150 $\mu\text{g/ml}$ ZnO NPs used in our experiment.
435 The same heterogeneity in the response of the MDA-MB-231 cells was observed after
436 exposure to ZnCl_2 . Incubation of the cells with ZnCl_2 resulted in an increase of FluoZin3
437 fluorescence (Figure 9C). The control elongated MDA-MB-231 cells, imaged under the same
438 conditions, survived the laser and did not show any increase in intracellular zinc throughout
439 the experiment, which confirms that the Zn^{2+} indicator did not fluoresce over time, in the
440 absence of zinc (Figure 9D). Figure 9E-F show the green fluorescent channel of FluoZin3-
441 Zn^{2+} complexes and red PI fluorescent channels inside the cells after 4 and 16 h exposure to
442 the bare ZnO NPs and ZnCl_2 . The MDA-MB-231 cells showed 54% (62/115 cells) and 100%
443 (115/115) death after 4 and 16 h incubation with the bare ZnO NPs, respectively (Figure 9E).
444 After exposure of the MDA-MB-231 cells to ZnCl_2 , 21 % of the cells (48/230) in the frame of
445 view were dead after 4 h, while 90 % of the cells (207/230) were dead after 16 h of exposure
446 (Figure 9F). Moreover, the distribution of Zn^{2+} was more diffuse inside the cytoplasm of the
447 cells exposed to ZnCl_2 (Figure 6C and 6F). The live imaging results for the control, ZnO and
448 ZnCl_2 incubations are in Supplementary information, Videos 2-4, respectively).

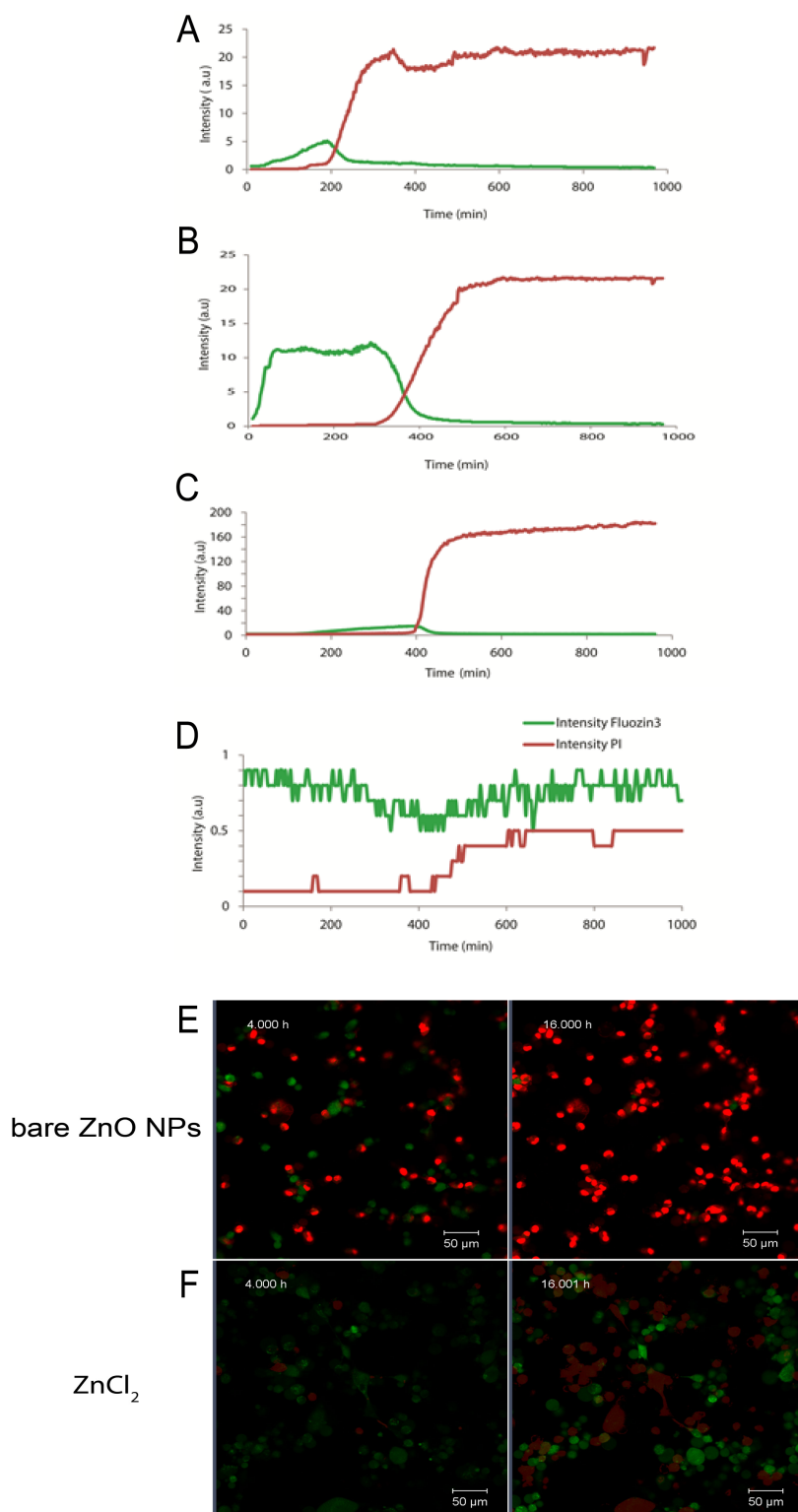
449 The kinetics of intracellular dissolution of the bare ZnO NPs and RGD-targeted ZnO
450 NPs were different: The bare ZnO NPs, showed a clear time dependent increase in
451 intracellular dissolution, while the RGD-targeted ZnO NPs showed low intracellular
452 dissolution initially and then a sudden burst of ionic zinc.

453

454

455

456



457

458 **Figure 9: (A-D) Cytoplasmic Zn²⁺ and PI concentration of MDA-MB-231 cells after exposure to the bare ZnO NPs**
 459 **and ZnCl₂ showing an increase in intracellular Zn²⁺ and subsequent cell death.** The MDA-MB-231 cells were loaded
 460 with the cell-permanent Zn²⁺ indicator FluoZin3-AM prior to incubation with (A) 150 μg/ml bare ZnO NPs, (B) 250 μg/ml
 461 ZnCl₂, or (D) imaging buffer (NA control). (E and F) Confocal images of MDA-MB-231 cells show the intracellular green
 462 fluorescent FluoZin3- Zn²⁺ and red PI and illustrate the heterogeneity of the cellular responses to ZnO and ZnCl₂,
 463 respectively. The MDA-MB-231 cells were loaded with the cell-permeant Zn²⁺ indicator FluoZin3-AM prior to incubation
 464 with 150 μg/ml of bare ZnO NPs or equivalent zinc ions concentration from ZnCl₂. FluoZin3 fluoresces green upon binding
 465 ionic zinc. Toxic levels of Zn²⁺ ions inside the cell resulted in loss of cell membrane integrity and cellular penetration of PI
 466 which stained the nuclei of dead cells red. Images were acquired over a time course of 16 h.

467 **3. Discussion**

468 TNBCs represent an important clinical challenge because these cancers do not respond to
469 endocrine therapy or other available targeted agents [37, 38]. Therefore, agents that
470 specifically target and kill these cells are of great importance. Compared with other NMs,
471 ZnO-based NPs are attractive due to their selective killing of cancer cells [18, 39]. The data
472 from the current study showed that although both bare and targeted ZnO NPs were toxic to
473 both the MDA-MB-231 TNBCs and MCF-7 (ER⁺) breast cancer cells, RGD targeting
474 enhanced uptake and induction of cell death of TNBCs. These findings support previous
475 work showing that the release of Zn²⁺ ions, following incubation of ZnO NPs with MCF-7
476 breast cancer epithelial cells, triggers the generation of reactive oxygen species, resulting in
477 damage to the plasma membrane [16]. The results also demonstrate, for the first time, that
478 ZnO-GFP-HCV-RGD NPs are internalized by TNBCs and show toxicity at lower doses
479 compared to the bare ZnO NPs after 24 h exposure. The higher toxicity of the RGD-targeted
480 ZnO NPs, could be due to a higher amount of internalization (and hence Zn²⁺ ion release) of
481 the targeted ZnO NPs through the integrin $\alpha\beta3$ receptors expressed by the MDA-MB-231
482 TNBCs compared to the bare ZnO NPs. This finding of the ability of the RGD-targeted ZnO
483 NPs to effectively kill TNBCs, which account for a high proportion of breast cancer mortality
484 due to the lack of effective therapies [64], will open new options for this material in cancer
485 therapy.

486 Only few reports on the cellular uptake of ZnO NMs are available [19, 23, 40-42].
487 Confocal microscopy has shown that ZnO NP remnants are internalised by caveolae mediated
488 uptake in BEAS-2B cells and toxic Zn²⁺ has been shown to accumulate in the lysosomal
489 compartment in RAW 264.7 cells [23]. ZnO NPs are rapidly internalized by HELA cells, and
490 dissolve in the lysosomal compartments [43]. Lin *et al.* showed agglomerates of ZnO NPs in
491 endosomes in bronchoalveolar carcinoma- derived cells (A549) after 24 h exposure to 12

492 $\mu\text{g/ml}$ ZnO NPs [44], however, their study lacks EDX data to confirm the presence of ZnO
493 NPs. There is still much debate around whether the ZnO NMs are taken up by cancer cells
494 and then dissolve only intracellularly or whether they dissolve in the extracellular matrix
495 releasing ionic zinc which diffuses into the cells. One study successfully directed fluorescent
496 ZnO NWs to tumours *in vivo* by targeting the integrin $\alpha\text{v}\beta\text{3}$ receptors [11] but reference to
497 whether the wires were internalised by the cells or to where the zinc dissolved and exerted its
498 toxic effects was not highlighted. Here, we show for the first time (using a Zn^{2+} -sensitive
499 dye) that in most cells, a rise of intracellular Zn^{2+} precedes cell death in the MDA-MB-123
500 TNBCs after exposure to ZnO NPs. Dissolution studies of the bare ZnO NPs in the absence
501 of cells, in the HEPES confocal imaging buffer at pH 7.4, showed that the release of ionic
502 zinc (Zn^{2+}) was $<9 \mu\text{g/ml}$ Zn^{2+} . Therefore, any rise in intracellular Zn^{2+} upon exposure to the
503 bare ZnO NPs is likely due to the acidic dissolution of the ZnO NPs inside the intracellular
504 vesicles and the local release of toxic Zn^{2+} ions.

505 The breast cancer cells showed inter- and intra- cellular heterogeneity in response to the
506 ZnO NPs. A marked variability between the cell responses of the MDA-MB-231 cells to the
507 ZnO-GFP-HCV-RGD NPs was measured. This variability may be due to several factors, such
508 as the number of ZnO-GFP-HCV-RGD NPs internalised by each cell and the route by which
509 they are internalised. Another key factor involves the potential variability of each cell within
510 the population to Zn^{2+} , which could be due to differences in cell function such as metabolism
511 and/or the phenotype of the individual cells. This heterogeneity of cancer cells introduces
512 significant challenges in designing effective treatment strategies. Inter-tumour heterogeneity
513 impacts significantly on stratifying patients, the selection of therapies and development of
514 targeted therapeutics. Intra-tumour heterogeneity has implications in the emergence of
515 resistance to treatment, identification of driver events, defining the phenotype of recurrent
516 disease and can impact the effectiveness of treatments when using drugs in combination [33].

517 Therefore, understanding and characterizing heterogeneity is crucial in the development of
518 refined treatment strategies. The ability of ZnO NPs to kill the TNBCs that could be resistant
519 to classic treatments while possessing potent tumor-forming capacity is another advantage of
520 using this material in cancer therapeutics. The observed heterogeneity in the response of the
521 breast cancer cells to the ZnO NPs could be explained since most cancers comprise a
522 heterogeneous population of cells with different proliferative and tumorigenic potentials [45,
523 46]. According to the recent cancer stem cell hypothesis, a tumor consists of differentiated
524 highly proliferative cells, and undifferentiated low-cycling cells with self-renewal capacity,
525 the so called cancer stem cells (CSCs) [47]. CSCs should have a higher resistance than the
526 differentiated cancer cells to radiation and chemotherapy [48]. The presence and the
527 percentage of these CSCs expressed by the MCF-7 and the MDA-MB-231 cells were
528 measured. Among the markers that are currently used to identify breast CSCs [49], the
529 expression of CD44, CD24, CD90, and EpCAM (epithelial cell adhesion molecule) were
530 tested. CSCs are reported as CD44⁺, CD24⁻, EpCAM^{low} and CD90⁻ [50]. In this study, the
531 MDA-MB-231 and the MCF-7 cells showed different expression of these markers
532 (Supplementary information, Figure S8); while the MCF-7 were CD44^{low}/CD24⁺/ EpCAM^{high}
533 and CD90⁺ (Supplementary information, Figure S8A), the MDA-MB-231 cells were
534 CD44⁺/CD24⁻/EpCAM^{low} and CD90⁻ (Supplementary information, Figure S8B). This
535 coincides with the published work for these cells [50, 51]. CD44⁺/CD24⁻ breast cancer cells
536 were reported to disseminate in the bone marrow and they were also resistant to
537 chemotherapy [52]. Therefore, the ability of ZnO NPs to eradicate these CSCs could further
538 support the therapeutic value of this material for the treatment of cancer.

539 The kinetics of intracellular dissolution of the bare ZnO NPs and RGD-targeted ZnO NPs
540 were different: The bare ZnO NPs, showed a clear time dependent increase in intracellular
541 dissolution, while the RGD-targeted ZnO NPs showed low intracellular dissolution initially

542 and then a sudden burst of ionic zinc, indicating that ZnO NP toxicity in both cell types is due
543 to a pH-triggered, intracellular release of ionic Zn^{2+} . This finding is most likely due to the
544 different mechanisms of internalisation of the bare and the RGD-targeted NPs and therefore
545 altered intracellular distribution [53]. It has been reported that the RGD-grafted NPs enter
546 cells through integrin or clathrin-mediated endocytosis and are localised in perinuclear
547 regions while the bare ZnO NPs are hypothesized to be taken up by the cells by clathrin-
548 independent endocytosis, and then end up in the acidic lysosomes of the cells where they
549 dissolve to release the zinc ions and labile zinc complexes [19, 23, 24, 54].

550 In conclusion, a nanoparticle platform was developed which is capable of delivering a
551 high local dose of Zn^{2+} ions from ZnO NMs intracellularly, coupled to simultaneous targeted-
552 delivery to destroy TNBCs. It is expected that this approach could be adapted to attach other
553 ligands for the future development of cell-targeted ionic nanoparticle therapeutics to treat
554 TNBCs. This is clinically promising since it has been reported that zinc may protect normal
555 human lymphocytes against DNA-damaging action and increase this action in human
556 myelogenous leukaemia K562 cancer cells [17], which indicates the dual action of this
557 element in dependency of target cells and can be useful in cancer therapy. More generally,
558 these observations highlight the need for dynamic and static characterization of NMs-cell
559 interactions at the cellular level in order to understand how cellular uptake and dissolution of
560 ZnO NMs can be correlated to the kinetics of cell death. Improving our understanding of how
561 targeted ZnO NMs interact with cells at the cellular level using both dynamic and quantitative
562 imaging techniques is paramount for appreciating the heterogeneity and dynamics of their
563 interactions with cancer cells and for extracting statistically relevant information at the
564 cellular level about these interactions. An improved understanding and quantitative
565 characterization of the heterogeneity of cancer cell response to nanomaterial therapeutics will
566 be crucial in the development of refined treatment strategies.

567 **4. Materials and Methods**

568 Bare ZnO NPs were purchased from SkySpring Nanomaterials, Inc., (product number
569 8410DL, Westhollow Drive, Houston, USA) at 99.8% purity. The powder was white to light
570 yellow in colour, and was stored at room temperature (RT) and vacuum sealed.

571 **Functionalization of the ZnO nanoparticles.** Cell targeting of the ZnO NPs was achieved
572 using an immobilization of an engineered protein which is composed of a cell targeting
573 peptide domain, a ZnO binding peptide domain and a green fluorescence protein (GFP) for
574 tracking the modified ZnO NPs by fluorescence microscopy. The cell targeting domain
575 comprises peptides with established cell-specific tumor targeting sequences (CDCRGDCFC)
576 [55]. The ZnO binding motif is a hexapeptide (HCVAHR) discovered using a
577 computationally assisted peptide design approach [56]. These two peptide domains were
578 connected via a glycine linker (GGGTRGGG) to ensure flexibility. The thrombin recognition
579 sequence (LVPRGS) was designed to be located between GFP and the zinc oxide binding
580 peptide to facilitate GFP cleavage from the HCV-RGD domain, if required (The protein is
581 abbreviated hereafter as GFP-HCV-RGD).

582 In order to engineer the fusion protein, synthetic DNA corresponding to HCV-RGD
583 domains with linker amino acids, and having restriction enzyme recognition sites (NcoI and
584 BamHI) was purchased from GenScript (Piscataway, NJ, UAS). This nucleotide sequence
585 was cloned into the C-terminus of the GFP gene in the pET-3a vector using NcoI and BamHI
586 restriction sites. The nucleotide sequence purchased from GenScript and the amino acid
587 sequence of the obtained GFP-HCV-RGD fusion protein is shown in Supplementary Figure
588 S1A and S1B, respectively. As a negative control for a cell-targeting assay, the RGD
589 sequence in the plasmid was changed to GGG using the established Agilent QuickChange
590 protocol.

591 The plasmids were then transformed and expressed in *E. coli* strain BL21 (DE3).
592 After culturing in LB medium at 37 °C with 100 mg/L ampicillin for 16 h, the cells were
593 pelleted from the media and resuspended in 50 mM HEPES, 100mM NaCl, pH 7.5 with a
594 protease inhibitor (complete Mini EDTA-free, Roche) and lysed using a DNase, RNase and
595 Lysozyme cocktail at r.t. for 30 minutes; followed by sonication. Cell debris was removed via
596 centrifugation, and supernatants were heat treated at 60°C for 10min. The heated solution was
597 re-centrifuged and the supernatant was dialyzed against 50 mM HEPES, 100 mM NaCl, pH
598 7.5 at 4 °C for overnight. The resulting supernatant was purified by size exclusion
599 chromatography (SEC) utilizing a Superose 6 column (GE Healthcare Life Sciences). The
600 protein elution was monitored by a UV-Vis detector at dual wavelengths of 280 nm (protein)
601 and 490 nm (GFP).

602 After preparing the engineered protein, it was diluted with the HEPES buffer (50 mM
603 HEPES, 100 mM NaCl, pH 7.5) to an absorbance of 1.6 at 490 nm. The bare ZnO NPs were
604 suspended in the same buffer at a concentration of 10 mg/ml. The ZnO NPs suspension was
605 diluted with the protein solution at a ratio of 1:9 vol/vol (*i.e.* concentration of the ZnO NPs is
606 1 mg/ml) and then incubated overnight at room temperature (RT) in the dark with gentle
607 shaking. Finally, the NPs dispersions were centrifuged at 13,000 rpm for five min on a table-
608 top centrifuge. To test the efficiency of binding of the targeting peptide (GFP-HCV-RGD)
609 and the control peptide (GFP-HCV-GGG) to ZnO NPs, the absorbance of the protein solution,
610 before and after incubation with the particles, was measured at 490 nm (Supplementary
611 information, Figure S2A and S2B, respectively).

612 ***Size and morphology of the zinc oxide nanoparticles.*** The morphology of bare and targeted
613 ZnO NPs was evaluated on a JEOL 2100F transmission electron microscope operated at 200
614 kV. A 3 µl droplet of NPs in MEM (10% FBS) solution was applied onto a 300 mesh holey-
615 carbon Cu grids (TAAB). Excess solution was blotted with filter paper and the grid was

616 plunged into liquid ethane using a Leica EM GP plunge freezer. The grid was then transferred
617 under liquid nitrogen into the microscope using a Gatan 914 high tilt liquid nitrogen cryo
618 transfer holder. Images were recorded at a magnification of 20000 X, with a defocus of -1 to
619 -2 μm , and under low dose conditions (a dose of 5 electrons per \AA^2 per second) with a Gatan
620 Orius SC1000 camera. The hydrodynamic size of NPs in MEM cell culture medium (10%
621 FBS) was characterized by dynamic light scattering using a Zetasizer Nano ZSP (Malvern
622 Instruments).

623 **Cell Culture.** Breast cancer cell lines (MCF-7 and MDA-MB231) were purchased from
624 ATCC (Manassas, VA, USA). The MDA-MB-231 and MCF-7 were maintained in DMEM
625 and MEM, respectively. DMEM and MEM media were supplemented with 10% fetal bovine
626 serum (FBS) and Penicillin/Streptomycin solution (100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$
627 streptomycin). Cells were maintained in a humidified incubator at 37°C and 5% CO_2 . In all
628 viability assays, cells were used from passages 5-25 and plated in flat-bottomed 96-well
629 plates (Fisher Scientific Ltd, UK). The MCF-7 cells were plated at a density of 3×10^5 cells
630 mL^{-1} (9×10^4 cells/ cm^2), while MDA-MB-231 were plated at a density of 2×10^5 cells mL^{-1}
631 (6×10^4 cells/ cm^2). The plates were incubated at 37°C and 5% CO_2 in a humidified incubator
632 for 24 hours. Then, supernatants from the culture plates were removed and fresh aliquots of
633 growth medium containing different concentrations of ZnO NPs (bare and targeted) were
634 added to the cells and then incubated with the cells for toxicity and IC_{50} analysis.

635 **Alamar blue assay.** Alamar Blue detects the impairment of cellular metabolism [57]. The
636 MDA-MB-231 and MCF-7 cells were exposed to targeted (10, 20, 25, 30, 40, and 50 $\mu\text{g}/\text{ml}$)
637 and bare (10, 20, 25, 30, 35, 40, 50, and 60 $\mu\text{g}/\text{ml}$) ZnO NPs, as well as ionic Zn^{2+} from
638 ZnCl_2 (equivalent to zinc ions released from 0, 10, 20, 25, 30 and 50 $\mu\text{g}/\text{ml}$ of ZnO) for 24 h.
639 The Alamar blue assay was used according to the manufacturer's protocol. The
640 spectrophotometric absorbance was measured at two wavelengths; 570 nm (reduced form of

641 Alamar Blue) and 600 nm (oxidized form of Alamar Blue) using a Bio-Rad plate-reader. The
642 mean absorbance reading from the wells without the particles was used as the control (100%
643 viability). The toxicity of the targeting peptide (GFP-HCV-RGD) to the cells was tested at
644 the highest concentration used in the assays (60 $\mu\text{g}/\text{ml}$). In this assay, the cells were registered
645 as viable, as long as they maintained their ability to reduce Alamar Blue. All experiments
646 were carried out in triplicate. The IC_{50} was generated from the dose-response curves for both
647 cell lines.

648 ***Lactate dehydrogenase (LDH) assay.*** LDH is a cytoplasmic enzyme that is released into the
649 supernatant upon cell lysis and measures membrane integrity [58]. The LDH released upon
650 exposure to ZnO NPs from damaged cells into the culture media was determined by a Takara
651 LDH Kit (Takara, CA, USA). The breast cancer cell lines were exposed to bare ZnO NPs (10,
652 20, 25, 30, 35, 40, 50, and 60 $\mu\text{g}/\text{ml}$) for 24 and 48 h. The assay was used according to the
653 manufacturer's protocol. The LDH assay requires a low-serum medium because FBS
654 contains LDH and can thus increase background absorbance [59]; therefore all culture media
655 used were supplemented with 1% FBS. The mean absorbance reading from the wells without
656 the particles was used as the negative control (100% viability), and those treated with 1%
657 Triton X-100 was used as a positive control (0% viability) [16]. Absorbance was measured
658 using a Bio-Rad plate-reader at 490 nm.

659 As the ZnO NPs could interact with dyes in the colorimetric assays, control
660 experiments using the Alamar Blue and LDH assays were performed using the same protocol
661 as the experiments in this study, but without the cells-in order to confirm that the ZnO NPs
662 did not interfere with the viability assays. No effects were observed for any of the assays.

663 ***ZnO nanoparticles binding assay.*** For all binding studies, cells were first cultured for 24
664 hours at 10^5 cells/ cm^2 . The cells were then washed twice and blocked for one hour in
665 blocking buffer (1% BSA in HBSS) prior to treatment to inhibit cellular uptake. Cells were

666 then treated for 30 minutes with 150 $\mu\text{g/ml}$ ZnO-GFP-HCV-RGD NPs in binding buffer (2
667 mM CaCl_2 , 1% BSA in HBSS) at 4°C. ZnO-GFP-HCV-RGD- treated cells were compared to
668 either untreated or ZnO-GFP-HCV-GGG NPs as negative controls. Prior to analysis, cells
669 were washed three times in binding buffer at 4°C.

670 For laser scanning confocal microscopy, cells were cultured on an 8-chamber cover
671 glass slide (Nunc, NY, USA; 0.19 mm thick, 0.8 cm^2/well) using the Zeiss LSM 710. A 488
672 nm laser line was used for excitation and images were analyzed using ImageJ software. Total
673 cell integrated density was calculated from representative confocal images of ZnO-GFP-
674 HCV-GGG and ZnO-GFP-HCV-RGD conditions. Total cell integrated density was corrected
675 to the background signal to give a corrected total cell fluorescence (CTCF) that was
676 normalized to cell area (CA) by dividing CTCF by CA, to preclude the cell size bias. 50 and
677 30 cells from ZnO-GFP-HCV-GGG and ZnO-GFP-HCV-RGD conditions were analyzed
678 respectively. To investigate the distribution of fluorescence intensity, representative confocal
679 images were divided into 100 tiles and integrated density was calculated.

680 For the fluorimetric based binding study, cells were cultured in 96-well black tissue
681 culture plate. After treatment, fluorescence intensity was measured using GloMax-Multi
682 detection system plate reader (Promega, WI, USA), using λ_{exc} of 490 nm and λ_{em} 510-570
683 nm filter. Fluorescence signal from untreated cells was used as a blank.

684 To determine the binding of ZnO-NP using FACS, cells were cultured in 6-well tissue
685 culture plate. Cells were excited using a 488 nm laser and emission was measured through
686 FL1 channel (530/30). At least 104 cells were quantified on a FACSCanto II flow cytometer
687 (BD bioscience, Franklin lakes, NJ) equipped with FlowJo 7.6 software.

688 ***Cell imaging by transmission electron microscopy:*** Cells were seeded on 6-well plates at a
689 density of 300k cells per well and exposed to NPs at a concentration of 30 $\mu\text{g/mL}$ for 3 h.
690 Following incubation, cells were rinsed briefly in saline (0.9% NaCl) to remove any non-

691 ingested particles and were then fixed in 2% gluteraldehyde (Sigma) and 2% formaldehyde
692 (Sigma) in 0.1M sodium cacodylate (Sigma) buffer at pH 7.4 for 1 h at room temperature.
693 The fixatives were then removed by washing cells with 0.1 M sodium cacodylate buffer 3
694 times. Cells were scraped and transferred into 1.5 mL Eppendorf tubes and cell pellets were
695 obtained by centrifugation at 10,000 g for 20 min. Samples were embedded without bulk
696 staining with osmium tetroxide. They were dehydrated in a graded ethanol series of 50%,
697 70%, 95%, and 100% (volume ratio of ethanol to DI-H₂O) ethanol for 5 min each then rinsed
698 three times in dry acetonitrile (Sigma) for an additional 10 min each, all at RT. After
699 dehydration, samples were progressively infiltrated with an Epon based resin, created by
700 combining 23.5 g Epoxy embedding medium (Sigma), 12.5 g dodecenyl succinic anhydride
701 (Agar scientific), 14 g methyl acid anhydride (Agar scientific), 0.75 mL benzyl
702 dimethylamine (Agar scientific). Samples were first re-suspended in 25% resin/acetonitrile
703 solution (volume ratio of resin to acetonitrile) and centrifuged for 30 s at 2,000 g. This step
704 was repeated with 50, 75, 100, 100 and 100% resin. Samples were cured at 100 °C for 2 h
705 [60]. Thin sections (70 nm) were cut directly into a water bath using an ultramicrotome with
706 a diamond knife with a wedge angle of 35 degrees. Sections were immediately collected on
707 bare, 250 mesh gold TEM grids (Agar Scientific), dried and kept under vacuum for TEM
708 analysis. HRTEM and HAADF-STEM/EDX analyses were performed on an FEI Titan 80–
709 300 scanning/transmission electron microscope (S/TEM) operated at 80 kV, fitted with a Cs
710 (image) corrector and a SiLi EDX spectrometer (EDAX, Leicester UK). STEM experiments
711 were performed with a convergence semi-angle of 14 mrad and inner and outer HAADF
712 collection angles of 49 and 239 mrad, respectively. The probe diameter was <0.5 nm.

713 ***Confocal Laser Scanning Microscopy.*** For live imaging, the MDA-MB-231 cells were
714 cultured for 48 h on 35 mm glass-based culture dishes (Nest Biotech Co, LTD, 35X12 mm
715 style, 20 mm glass bottom) at 6×10^4 cells/cm². The cells were rinsed once with the HEPES

716 confocal imaging buffer at pH 7.4 and 37°C. The cells were then loaded with 5 μ M of the
717 cell-permeant Zn^{2+} indicator FluoZin3-AM (Invitrogen, UK) in 1 ml of the HEPES confocal
718 imaging buffer for 20 min at 37°C for the bare ZnO NPs and $ZnCl_2$, or with 10 μ M of the
719 cell-permeant Zn^{2+} indicator Zinquin-AM in 1 ml of the HEPES confocal imaging buffer for
720 30 min at 37°C for the ZnO-GFP-HCV-RGD NPs. After rinsing the cells twice with imaging
721 buffer at 37°C to remove any dye that is non-specifically associated with the cell surface, the
722 cells were incubated for a further 30 min to allow complete de-esterification of intracellular
723 AM esters of Zinquin or FluoZin3. PI (20 μ g/ml) was prepared in 1 ml HEPES confocal
724 imaging buffer and added to the cells (the final concentration incubated with the cells was 10
725 μ g/ml). Then the bare or the targeted ZnO NPs were prepared in 1 ml HEPES imaging buffer
726 and were added to the cells just before imaging (final concentration exposed to the cells is
727 150 μ g/ml). $ZnCl_2$ was used at a concentration of 250 μ g/ml. This concentration of $ZnCl_2$ was
728 chosen as it has the same Zn^{2+} concentration as 150 μ g/ml ZnO. Confocal live imaging was
729 started immediately after addition of the ZnO NPs and $ZnCl_2$ to the cells using a Zeiss LSM
730 710 confocal microscope with the environmental chamber set to 37°C. Control MDA-MB-
731 231 cells were incubated with 1 ml of the HEPES confocal imaging buffer alone. For the bare
732 ZnO NPs and $ZnCl_2$, the 488 nm laser line was used to excite FluoZin3 ($\lambda_{exc}/\lambda_{em}$ = 494/516
733 nm) and the 561 nm laser was used to excite PI ($\lambda_{exc}/\lambda_{em}$ = 538/617 nm). Images were
734 acquired over a time period of 16 h (1 frame/2 min). For the ZnO-GFP-HCV-RGD, the 405
735 nm laser line was used to excite Zinquin ($\lambda_{exc}/\lambda_{em}$ = 344/385 nm), the 561 nm laser was
736 used to excite PI ($\lambda_{exc}/\lambda_{em}$ = 538/617 nm) and the 488 nm laser was used to excite GFP
737 labelled ZnO-HCV-RGD ($\lambda_{exc}/\lambda_{em}$ =488/509 nm). Since the dissolution of the targeted ZnO
738 NPs was slow, images were acquired over a time period of 24 h and at lower frequency (1
739 frame/4 min) to reduce the damaging effect of the laser. A 20x objective/0.8 NA was used

740 and the pinhole was set at 1 AU. Images and fluorescence quantification were carried out
741 using ZEN 2009 Confocal Software.

742 ***Confocal imaging processing:*** One z-plane location was extracted from the stack, and the
743 full image sequence from that depth slice was stored in LSM format. Data were loaded into
744 *Matlab* Version 2014b (The Mathworks, Inc) as unsigned 8-bit data. The data were found to
745 be interleaved, with odd and even-numbered frames. The odd-numbered frames contained the
746 data of interest (color channels). The odd-numbered frames were extracted from the LSM
747 file, and the red, green and blue frames extracted, together with bright-field images. The cells
748 were clearly visible on the bright-field images (Supplementary information, Figure S7). Some
749 motion and deformation of cells during the sequence was observed. This motion was faster at
750 the beginning of the sequence, slower towards the end. An algorithm was written to track
751 specific locations on the cell throughout frames, allowing local average intensities to be
752 obtained in a cell-relative manner.

753 ***Cell Selection and Region Selection:*** In the analysis, cells were selected that were not
754 clumped together, reducing the probability of “cross-talk” in the captured color channels.
755 Three locations were selected to act as the centers for sampling color channel intensity: a) on
756 the boundary of cells (R1), b) 75% of the distance between the cell’s center and boundary
757 location (R2), and b) within the center of a cell (R3). The locations were selected to be easy
758 to track, containing some spatial structure, and were placed manually by a naïve observer.
759 The sampling of the intensities was performed by placing a 15x15 Gaussian-weighted
760 window function centered at the selected locations. This was used to construct weighted
761 intensity samples from the three regions sampled within each cell. The Gaussians were all
762 unit normalized in integral.

763 ***Cell Region Tracking:*** Within individual cells, regions would deform over the sequence,
764 making absolute image coordinates unreliable as locations for sampling the color signal

765 channels. Therefore, we developed and applied a region tracking algorithm to “follow”
766 regions through frames. The region tracking used spatial patterns in smoothed versions of the
767 light-field images, and was based on a pixel level algorithm that used patch cross-correlation.
768 The patches were pre-filtered with either Gaussian or Laplacian of Gaussian functions
769 (depending on the nature of the local bright field image structure), and tracking was verified
770 by eye. If tracking failed, new locations were taken on the boundary to be tracked. The
771 tracked region centroids were used as the centers for the Gaussian-weighted sampling
772 regions.

773 ***Correlation of PI and ionic zinc inside and outside the cells:*** To assess the correlation
774 between levels of Propidium iodide and ionic zinc, we calculated the absolute value of the
775 correlation coefficient (ρ) between the red (PI) and blue (Zinquin) signal levels. Signals were
776 first normalized to the maximum value over the respective colour channels (red, green, blue)
777 observed within the ten cells that were analysed. A bootstrap technique was used to estimate
778 the probability distributions of the absolute value (unsigned) of the correlation coefficient, ρ ,
779 between red and blue signal levels, and then to evaluate the hypothesis that the levels of
780 Propidium iodide and ionic zinc were strongly correlated (H_1). For each cell, 100 samples
781 were drawn with replacement at random times from the blue and red channel signals within
782 the centre of randomly selected (with replacement) cells. The correlation coefficient was
783 calculated between these sample pairs. This process was repeated 10,000 times, to create an
784 estimate of the distribution of $|\rho|$. Samples (again, 10,000, with replacement of cells and time
785 samples) were also drawn from a surrogate null hypothesis (H_0) set of data that were obtained
786 by randomly shuffling the time axis of observations. The distributions are displayed through
787 the probability density plots for null hypothesis and under the hypothesis of strong
788 correlation. The 20,000 correlation values are also summarized within a box plot, indicating
789 median absolute correlation coefficient (red lines), 25th and 75th percentiles (box edges) and

790 approximately 99% coverage of the values of absolute correlation coefficient. Thus, the
791 distributions of time-paired (H_1) and unpaired (H_0) distributions are very distinct. This
792 suggests highly significant correlation (Kolmogorov-Smirnov statistic of 0.97) between ionic
793 zinc levels and Propidium iodide concentrations from the same locations within cells. We
794 tested image regions outside of cells for correlation between red and blue signals, to eliminate
795 the possibility of correlation being present within the same images, but outside of cells. The
796 same bootstrap test was performed on 8 regions outside of cells, including some with no
797 visible structures within the regions, and others containing zinc particles. The same absolute
798 correlation coefficient (ρ) was calculated between time-shuffled and unshuffled data. There is
799 no discernible difference in the distributions (KS-statistic of 0.03). This indicates that we can
800 reject the hypothesis that the observed correlation between red and blue channels is in some
801 way present within the imaging system: it clearly is to be found only within the cells.

802 **Statistical analysis.** All data were expressed as the mean \pm standard deviation of the values
803 obtained from three replicates. Statistical analysis was carried out using a paired Student's t -
804 test in all the viability assays. One way analysis of variance (ANOVA) was used to determine
805 the statistical significance in the Annexin V/7AAD assay. The level of significance was
806 accepted at $p \leq 0.05$. The level of significance was indicated by the Asterisks: * $p < = 0.05$.
807 The KS Statistic used for Figure 8 is a non-parametric test statistic that compares the shapes
808 of two distributions to see if they are close or very far apart. Large values of KS statistic
809 (close to 1) indicate very large differences in distribution. Small value (close to 0) indicates
810 that two distributions are quite similar. The reference distributions representing the null
811 hypothesis (H_0) are data of the same amplitude distributions as the raw data, but with
812 randomized time axes. Therefore where the KS statistic is large, the data are strongly
813 correlated in time (H_1); where the KS statistic is small, the data are uncorrelated in time.

814

815 **ACKNOWLEDGEMENTS**

816 A special thank you to Dr. Rachid Sougrat and Dr. Wei Zhu from the KAUST Imaging and
817 Characterization Core lab for their immense expertise in confocal imaging and electron
818 microscopy and also to Dr. Tahir F. Yapici from the KAUST Analytical Core lab for his
819 skilled technical support. We also thank the members of the Cell Migration and Signaling lab
820 at KAUST for their support.

821 **FUNDING**

822 This effort was supported by a King Abdullah University of Science and Technology Faculty
823 Baseline Research Funding Program (BAS) to J.S.M. and was also supported by a Global
824 Collaborative Research (AEA) Grant awarded to J.S.M., A.E.P. and M.R. A.E.P
825 acknowledges funding from an Individual ERC starting grant (CNTBBB). The funders had
826 no role in study design, data collection and analysis, decision to publish or preparation of the
827 manuscript.

828 **COMPETING INTEREST**

829 No conflict of interest is declared.

830 References

- 831 1. Holliday, D.L. and V. Speirs, *Choosing the right cell line for breast cancer research*. Breast
832 Cancer Research, 2011. **13**(4).
- 833 2. Brenton, J.D., et al., *Molecular classification and molecular forecasting of breast cancer:
834 Ready for clinical application?* Journal of Clinical Oncology, 2005. **23**(29): p. 7350-7360.
- 835 3. von Minckwitz, G. and M. Martin, *Neoadjuvant treatments for triple-negative breast cancer
836 (TNBC)*. Annals of Oncology, 2012. **23**: p. 35-39.
- 837 4. Sirohi, B., et al., *Platinum-based chemotherapy in triple-negative breast cancer*. Annals of
838 Oncology, 2008. **19**(11): p. 1847-1852.
- 839 5. McNeil, S.E., *Nanotechnology for the biologist*. J Leukoc Biol, 2005. **78**(3): p. 585-94.
- 840 6. Banerjee, H.N. and M. Verma, *Application of nanotechnology in cancer*. Technology in
841 Cancer Research & Treatment, 2008. **7**(2): p. 149-54.
- 842 7. Nel, A.X., T. Madler, L. Li, N., *Toxic potential of materials at the nanolevel*. Science, 2006.
843 **311**(5761): p. 622-627.
- 844 8. Kishwar, S., et al., *Intracellular ZnO Nanorods Conjugated with Protoporphyrin for Local
845 Mediated Photochemistry and Efficient Treatment of Single Cancer Cell*. Nanoscale Research
846 Letters, 2010. **5**(10): p. 1669-1674.
- 847 9. Zhang, H., et al., *A strategy for ZnO nanorod mediated multi-mode cancer treatment*.
848 Biomaterials, 2011. **32**(7): p. 1906-1914.
- 849 10. Mitra, S., et al., *Porous ZnO nanorod for targeted delivery of doxorubicin: in vitro and in vivo
850 response for therapeutic applications*. Journal of Materials Chemistry, 2012. **22**(45): p.
851 24145-24154.
- 852 11. Hong, H., et al., *Cancer-targeted optical imaging with fluorescent zinc oxide nanowires*. Nano
853 Letters, 2011. **11**(9): p. 3744-50.
- 854 12. Prasad, A.S., *Zinc in human health: Effect of zinc on immune cells*. Molecular Medicine, 2008.
855 **14**(5-6): p. 353-357.
- 856 13. Vallee, B.L. and D.S. Auld, *Zinc coordination, function, and structure of zinc enzymes and
857 other proteins*. Biochemistry, 1990. **29**(24): p. 5647-5659.
- 858 14. Dineley, K.E., T.V. Votyakova, and I.J. Reynolds, *Zinc inhibition of cellular energy production:
859 implications for mitochondria and neurodegeneration*. Journal of Neurochemistry, 2003.
860 **85**(3): p. 563-570.
- 861 15. Franklin, R.B. and L.C. Costello, *The Important Role of the Apoptotic Effects of Zinc in the
862 Development of Cancers*. Journal of Cellular Biochemistry, 2009. **106**(5): p. 750-757.
- 863 16. Sasidharan, A., et al., *Rapid dissolution of ZnO nanocrystals in acidic cancer
864 microenvironment leading to preferential apoptosis*. Nanoscale, 2011. **3**(9): p. 3657-3669.
- 865 17. Sliwinski, T., et al., *Zinc salts differentially modulate DNA damage in normal and cancer cells*.
866 Cell Biology International, 2009. **33**(4): p. 542-547.
- 867 18. Hanley, C., et al., *Preferential killing of cancer cells and activated human T cells using ZnO
868 nanoparticles*. Nanotechnology, 2008. **19**(29).
- 869 19. Muller, K.H., et al., *pH-dependent toxicity of high aspect ratio ZnO nanowires in
870 macrophages due to intracellular dissolution*. ACS Nano, 2010. **4**(11): p. 6767-79.
- 871 20. Rasmussen, J.W., et al., *Zinc Oxide Nanoparticles for Selective Destruction of Tumor Cells and
872 Potential for Drug Delivery Applications*. Expert Opinion Drug Delivery, 2010. **7**(9): p. 1063-
873 77.
- 874 21. Tannock, I.F. and D. Rotin, *Acid Ph in Tumors and Its Potential for Therapeutic Exploitation*.
875 Cancer Research, 1989. **49**(16): p. 4373-4384.
- 876 22. David, C.A., et al., *Dissolution Kinetics and Solubility of ZnO Nanoparticles Followed by
877 AGNES*. Journal of Physical Chemistry C, 2012. **116**(21): p. 11758-11767.
- 878 23. Xia, T., et al., *Comparison of the Mechanism of Toxicity of Zinc Oxide and Cerium Oxide
879 Nanoparticles Based on Dissolution and Oxidative Stress Properties*. ACS Nano, 2008. **2**(10):
880 p. 2121-2134.

- 881 24. Gilbert, B., et al., *The Fate of ZnO Nanoparticles Administered to Human Bronchial Epithelial*
882 *Cells*. ACS Nano, 2012. **6**(6): p. 4921–4930.
- 883 25. Gasparini, G., et al., *Vascular integrin alpha(v)beta3: a new prognostic indicator in breast*
884 *cancer*. Clinical Cancer Research, 1998. **4**(11): p. 2625-34.
- 885 26. Brooks, P.C., R.A.F. Clark, and D.A. Cheresh, *Requirement of Vascular Integrin*
886 *Alpha(V)Beta(3) for Angiogenesis*. Science, 1994. **264**(5158): p. 569-571.
- 887 27. Rathinam, R. and S.K. Alahari, *Important role of integrins in the cancer biology*. Cancer
888 *Metastasis Reviews*, 2010. **29**(1): p. 223-37.
- 889 28. Qamar, S., et al., *Anticancer SAR models for MCF-7 and MDA-MB-231 breast cell lines*.
890 *Anticancer research*, 2011. **31**(10): p. 3247-3252.
- 891 29. Boyd, M.R., *The NCI in vitro anticancer drug discovery screen*, in *Anticancer Drug*
892 *Development Guide*. 1997, Springer. p. 23-42.
- 893 30. Monks, A., et al., *Feasibility of a high-flux anticancer drug screen using a diverse panel of*
894 *cultured human tumor cell lines*. Journal of the National Cancer Institute, 1991. **83**(11): p.
895 757-766.
- 896 31. Chen, S., et al., *Avoiding artefacts during electron microscopy of silver nanomaterials*
897 *exposed to biological environments*. Journal of microscopy, 2015.
- 898 32. Mu, Q.S., et al., *Systematic Investigation of the Physicochemical Factors That Contribute to*
899 *the Toxicity of ZnO Nanoparticles*. Chemical Research in Toxicology, 2014. **27**(4): p. 558-567.
- 900 33. Zardavas, D., et al., *Clinical management of breast cancer heterogeneity*. Nature Reviews
901 *Clinical Oncology*, 2015. **12**(7): p. 381-94.
- 902 34. Qin, Y., et al., *Measuring steady-state and dynamic endoplasmic reticulum and Golgi Zn(2+)*
903 *with genetically encoded sensors*. Proceedings of the National Academy of Sciences of the
904 United States of America, 2011. **108**(18): p. 7351-7356.
- 905 35. Rounds, C.M., et al., *Propidium iodide competes with Ca(2+) to label pectin in pollen tubes*
906 *and Arabidopsis root hairs*. Plant Physiol, 2011. **157**(1): p. 175-87.
- 907 36. Krzywinski, M. and N. Altman, *Points of significance: Visualizing samples with box plots*.
908 *Nature methods*, 2014. **11**(2): p. 119-120.
- 909 37. Cleator, S., W. Heller, and R.C. Coombes, *Triple-negative breast cancer: therapeutic options*.
910 *The Lancet Oncology*, 2007. **8**(3): p. 235-244.
- 911 38. Hudis, C.A. and L. Gianni, *Triple-negative breast cancer: an unmet medical need*. The
912 *oncologist*, 2011. **16**(Supplement 1): p. 1-11.
- 913 39. Akhtar, M.J., et al., *Zinc oxide nanoparticles selectively induce apoptosis in human cancer*
914 *cells through reactive oxygen species*. International journal of nanomedicine, 2012. **7**: p. 845.
- 915 40. Bacchetta, R., et al., *Evidence and uptake routes for Zinc oxide nanoparticles through the*
916 *gastrointestinal barrier in Xenopus laevis*. Nanotoxicology, 2014. **8**(7): p. 728-44.
- 917 41. Jingwen, S., et al., *Microsomal Glutathione Transferase 1 Protects Against Toxicity Induced*
918 *by Silica Nanoparticles but Not by Zinc Oxide Nanoparticles*. ACS Nano, 2012.
- 919 42. Churchman, A.H., et al., *Serum albumin enhances the membrane activity of ZnO*
920 *nanoparticles*. Chemical communications (Cambridge, England), 2013. **49**(39): p. 4172-4174.
- 921 43. Muhammad, F., et al., *pH-Triggered controlled drug release from mesoporous silica*
922 *nanoparticles via intracellular dissolution of ZnO nanolids*. Journal of the American Chemical
923 Society, 2011. **133**(23): p. 8778-81.
- 924 44. Lin, W.S., et al., *Toxicity of nano- and micro-sized ZnO particles in human lung epithelial cells*.
925 *Journal of Nanoparticle Research*, 2009. **11**(1): p. 25-39.
- 926 45. Al-Hajj, M., et al., *Therapeutic implications of cancer stem cells*. Current Opinion in Genetics
927 & Development, 2004. **14**(1): p. 43-47.
- 928 46. Selby, P., J.P. Bizzari, and R.N. Buick, *Therapeutic implications of a stem cell model for human*
929 *breast cancer: a hypothesis*. Cancer Treatment Reports, 1983. **67**(7-8): p. 659-63.
- 930 47. Greve, B., et al., *Flow cytometry in cancer stem cell analysis and separation*. Cytometry A,
931 2012. **81**(4): p. 284-93.

- 932 48. Kakarala, M. and M.S. Wicha, *Implications of the cancer stem-cell hypothesis for breast*
933 *cancer prevention and therapy*. Journal of Clinical Oncology, 2008. **26**(17): p. 2813-2820.
- 934 49. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells*. PNAS,
935 Proceedings of the National Academy of Sciences, 2003. **100**(7): p. 3983-8.
- 936 50. Prat, A., et al., *Phenotypic and molecular characterization of the claudin-low intrinsic subtype*
937 *of breast cancer*. Breast Cancer Research, 2010. **12**(5).
- 938 51. Sheridan, C., et al., *CD44(+)/CD24(-) breast cancer cells exhibit enhanced invasive properties:*
939 *an early step necessary for metastasis*. Breast Cancer Research, 2006. **8**(5): p. 13.
- 940 52. Burdick, M.M., et al., *Expression of E-selectin ligands on circulating tumor cells: cross-*
941 *regulation with cancer stem cell regulatory pathways?* Frontiers in Oncology, 2012. **2**: p. 103.
- 942 53. Danhier, F., A. Le Breton, and V. Preat, *RGD-Based Strategies To Target Alpha(v) Beta(3)*
943 *Integrin in Cancer Therapy and Diagnosis*. Molecular Pharmaceutics, 2012. **9**(11): p. 2961-
944 2973.
- 945 54. Adam, N., et al., *The chronic toxicity of ZnO nanoparticles and ZnCl₂ to Daphnia magna and*
946 *the use of different methods to assess nanoparticle aggregation and dissolution*.
947 Nanotoxicology, 2014. **8**(7): p. 709-717.
- 948 55. Arap, W., R. Pasqualini, and E. Ruoslahti, *Cancer treatment by targeted drug delivery to*
949 *tumor vasculature in a mouse model*. Science, 1998. **279**(5349): p. 377-380.
- 950 56. Okochi, M., et al., *Peptide array-based characterization and design of ZnO-high affinity*
951 *peptides*. Biotechnology Bioengineering, 2010. **106**(6): p. 845-51.
- 952 57. Rampersad, S.N., *Multiple Applications of Alamar Blue as an Indicator of Metabolic Function*
953 *and Cellular Health in Cell Viability Bioassays*. Sensors, 2012. **12**(9): p. 12347-12360.
- 954 58. Holder, A.L., et al., *Particle-induced artifacts in the MTT and LDH viability assays*. Chemical
955 Research in Toxicology, 2012. **25**(9): p. 1885-92.
- 956 59. Kimura, Y., et al., *Hydrogen sulfide protects HT22 neuronal cells from oxidative stress*.
957 Antioxidants & Redox Signaling, 2006. **8**(3-4): p. 661-670.
- 958 60. McDonald, K.L., *Rapid embedding methods into epoxy and LR White resins for morphological*
959 *and immunological analysis of cryofixed biological specimens*. Microscopy and microanalysis,
960 2014. **20**(01): p. 152-163.
- 961