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Tracking cell proliferation using a nanotechnology based approach

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ABSTRACT

Aim: To develop an efficient nanotechnology fluorescence based method to track cell proliferation to avoid the limitations of current cell-labelling dyes. **Material & methods:** Synthesis, PEGylation, bifunctionalization and labelling with a fluorophore (Cy5) of 200 nm polystyrene nanoparticles (NPs) were performed. These NPs were characterised and assessed for in vitro long-term monitoring of cell proliferation. **Results:** The optimisation and validation of this method to track long term cell proliferation assays have been achieved with high reproducibility, without cell cycle disruption. This method has been successfully applied in several adherent and suspension cells including hard-to-transfect cells and isolated human primary lymphocytes. **Conclusion:** A novel approach to track efficiently cellular proliferation by flow cytometry using fluorescence labelled nanoparticles has been successfully developed.

KEYWORDS

Fluorescent nanoparticles, cell proliferation, cell tracking, flow cytometry, nanofection

Introduction

Cell division, as the default status, is a *sine qua non* condition for life [1]. In normal cells, the cell growth and division processes are tightly controlled by complex mechanisms [2]. Many biological responses are related to changes in cell proliferation. For instance the abnormal and uncontrolled cell proliferation is a hallmark of cancer cells [3], being crucial cell proliferation assays to control the division rate in cancer research. By contrast, the lack of proliferative ability of immune cells may indicate an immunodeficiency disorder. A key feature of the adaptive immunity is the T-cell proliferative responses to antigen stimuli [4]. Therefore, the measure of cell proliferation is essential in the assessment of the immune status. Among the wide variety of methods for measuring dividing cells, cell-labelling fluorescent dyes for monitoring proliferation are commercially available [5]. These exogenous reporters are chemical entities which interact with cellular components such as cell membrane (e.g., PKH lipophilic dyes), cytoplasm (e.g., CFSE) and nucleus (e.g., Hoechst 33342), and are distributed to daughter cells after each cell division, in a theoretically equal distribution, resulting in a progressive halving of the progeny fluorescence. This fluorescence intensity reduction can be quantified by conventional fluorescent techniques, the most used one being flow cytometry [5,6]. Although these fluorescent reporters are one of the most common used by the scientific community for the measurement of cell proliferation, they present some limitations such as alteration of the normal function of tracked cells, uneven distribution within the progeny population, rapid dilution during cell proliferation and high cytotoxic effects [5,6]. Consequently, there is a need for a safe and efficient alternative method to track successfully cell proliferation to overcome these limitations.

Over the last decade, our group has developed several nanotechnologies for preparing functionalised polystyrene nanoparticles (NPs) which are then conjugated to cargoes of different nature from small molecules (fluorophores, sensors, small drugs) to biomolecules (proteins and nucleic acids and their mimics). The main benefits of using polystyrene nanoparticles are cellular environment is not degraded and there is no apparent toxicity to cells even in long term studies [7]. Specifically surface-modified polystyrene nanoparticles are homogeneous, exhibit a low polydispersity index, and form stable colloids in biological fluids. These nanodevices have been extensively used as systems for *in vitro* applications [7–9]. Furthermore, these NPs enable solid phase multistep chemistries to become compatible with different bio-orthogonal strategies [10,11]. Easy entry in a broad range of cell types including adherent, suspensions and primary cell lines has been reported [8–12]. On the other hand, gene-expression profiling studies showed that these NPs did not induce any significant alteration in nanofected cell transcriptomes [13]. Recent proteomic studies showed no key regulators of cell cycle were affected by the internalisation of these NPs, resulting in a parent-progeny transfer of the nanofection load. Furthermore, NPs are not exported from the cells because externalization- exocytosis rate is negligible, allowing long-term monitoring [14], and their intracellular localization has been proved [15]. These properties make NPs ideal to be used as cell proliferation devices.

Herein, we present a novel approach to monitor cell proliferation based on the use of fluorescent bifunctionalised crosslinked polystyrene NPs (referred as fluorescent NPs). The alternative method is inspired by the use of cell-labelling dyes to quantify cell proliferation by fluorescence techniques but avoiding the limitations of the mentioned dyes by applying nanotechnology.

Materials & methods

Cell Culture

Cell lines were provided by the cell bank of the CIC of the University of Granada. For this study we used: as model of adherent cells, the human breast cancer cell lines: MCF-7, MDA-MB-468 and MDA-MB-231; as model of suspension cells, the lymphoma cell line Raji (B-lymphoblastic) and two types of leukemia cell lines: K562 (erythroleukemic) and Jurkat (T-lymphoblastic). Adherent cell lines were cultured in DMEM base medium (Gibco) and suspension cell lines in RPMI base medium (Gibco) supplemented with 10% (vol/vol) Fetal Bovine Serum (Gibco), 1% L-Glutamine (Gibco) and 1% Penicillin/Streptomycin (Gibco) in a humidified incubator at 5% CO₂ and 37° C. All cell lines tested negative for mycoplasma infection.

Preparation of Fluorescent nanoparticles (NPs)

200 nm (PDI 0.089) amino functionalized polystyrene crosslinked nanoparticles were prepared by dispersion polymerisation following the protocol previously described [16]. PEGylation, bifunctionalization and dye conjugation were performed following a Fmoc-Dde orthogonal strategy using Oxyma/DIC as coupling reagents (see **Supplementary Fig. 1 and Supplementary information**). The loading (mmol/g free amino groups) was calculated by conjugation of Fmoc-Glycine and quantification of Fmoc release analysed by UV spectrophotometry. The effectiveness of the Cy5 conjugation was checked by flow cytometry with a FACSCanto II flow cytometer (Becton Dickinson & Co., NJ, USA) and by fluorescence microscopy with Confocal Scanning Microscope Zeiss LSM 710 (**Supplementary Fig. 1**). As control Fmoc deprotected PEGylated 200 nm NPs, referred as naked NPs, were used. Dynamic Light Scattering (DLS) and Zeta potential were measured on a Zetasizer Nano ZS ZEN 3500 in molecular biology grade water in a disposable sizing

cuvette for hydrodynamic size measurements or clear disposable zeta cuvette for zeta potential measurements. The stability of nanoparticles at 37 °C in culture medium was tested at different time points by measuring the hydrodynamic size and zeta potential (see **(Supplementary Table 1)**).

Fluorescent NPs uptake study by flow cytometry

Adherents and suspension cell lines were incubated with the fluorescent NPs at the established incubation times in a humidified incubator at 5% CO₂ and 37° C. Naked NPs were used as control (at the specific ratio cell/NPs), and cells without nanoparticles treatment. After that, for adherent cells, they were detached and washed with PBS 1X; for suspension cells, the media was removed by centrifugation and washed once with PBS 1X. In the case of suspension hard-to-transfect cells, additional wash step adding a reducing agent (tris(2-carboxyethyl)phosphine, Fluorochem) was done in order to quench possible binding nanoparticles in the membrane extracellular side, avoiding fluorescence interference from unincorporated adsorbed NPs. Then, samples were fixed in 2% paraformaldehyde (PFA) and analyzed via flow cytometry using FACSCanto II flow cytometer (Becton Dickinson & Co., NJ, USA). See Supplementary information for detailed protocol.

Fluorescent nanoparticle-based proliferation assay

Cells were properly disposed to nanoparticle incubation as described above, using 1:25000 cell:NPs ratio and 30 minutes of nanofection. Immediately after the incubation had finished, a sample of nanofected cells was fixed and named time 0. After that, the rest of the nanofected cells were plated and maintained using appropriate culture conditions for each cell line. Every day, a sample of the nanofected cells was fixed and named as the corresponding harvesting

day (day 1, 2, 3, 4, 5, 6 and 7). Untreated and arresting cells treated with 0.5 µg/mL of mitomycin C (Sigma) were used as a control. Once the assay was finished, all the samples were analyzed by flow cytometry. Data acquisition and analysis was performed using the BP 660/20 nm (APC filter) on a BD FACSCanto II Flow Cytometer with BDFACSDiva™61 software. Harvest cells from culture wells can also be analyzed directly without fixation step by flow cytometry for cell proliferation.

In vitro lymphocyte proliferation assay

Peripheral blood mononuclear cells (PMBCs) were thawed according to manufacture instructions in order to collect lymphocyte population. Nanofection protocol was adapted from suspension hard-to-transfect cells proliferation assay. See Supplementary information for detailed protocol. Appropriate stimuli to induce proliferation of lymphocyte subsets, phytohemagglutinin (PHA-P; Sigma), was used as a polyclonal T-cell mitogen at 2%, and proliferation was analysed by flow cytometry. CFSE assay was performed according to the manufacturer's instructions as a control to monitor proliferation lymphocyte model assay.

Results

Optimisation of the method

A monodispersed population of 200 nm amino functionalized cross-linked PS-NPs (PDI 0.089) ((1) Supplementary Fig. 1a) was obtained by dispersion polymerization as previously described [16]. NPs were firstly functionalized with a polyethylene glycol (PEG) spacer PEGylation of PS-NPs ((2) Supplementary Fig. 1a) was performed following a Fmoc solid phase protocol and using Oxyma/DIC as coupling reagents. This PEGylation increases the

biocompatibility of the NPs, thereby facilitating their transport across cell membranes. The NPs were bifunctionalized following an orthogonal strategy based on the use of 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl (Dde) and Fluorenylmethyloxycarbonyl (Fmoc) protecting groups [17]. This bifunctionalization allows the labelling of the nanoparticle by conjugation of a near infrared fluorophore (Cy5) (activated as NHS ester) while keeping a defined amount of free amino groups to keep the positive surface charge of the nanoparticle. The stability of these nanoparticles was tested. These nanoparticles are stable at storage conditions (4°C, 2% solid content in water). In another hand, the stability at incubation conditions, (37 °C in culture media supplemented with serum) has been tested (see **Supplementary Fig. 1, Supplementary Table 1 and Supplementary information** for details of synthesis and characterization and stability studies).

Considering the requirements of fluorescent dye labelling procedures, the number of fluorescent NPs per cell and the incubation time required for obtaining a 100% of nanofected cells were calculated. The number of fluorescent NPs was calculated using a spectrometric based method recently reported by us [18].

To optimize cellular uptake, three different ratios of fluorescent NPs per cell (1:12500, 1:25000 and 1:50000; cells: fluorescent NPs) were interrogated at a fixed incubation time of 60 minutes using human breast cancer cell lines: MCF-7, MDA-MB-468 and MDA-MB-231. Following incubation, cells were analyzed by flow cytometry and the percentage of cells which are nanofected -nanofection percentage- was calculated (**Fig. 1a**). The data show that although there are high percentage of cells nanofected in all tested conditions, the required 100% value was reached when using the 1:25000 ratio (cell:fluorescent NPs). Although 100% of cells are nanofected at one point, NPs uptake continues and the nanofection load increases proportionally to the number of NPs. For this reason, an analysis of the median of

fluorescence intensity increments ($\Delta\text{MFI} = \text{MFI}_{\text{nanofected}} / \text{MFI}_{\text{non-nanofected}}$) was performed to obtain information about nanofection load (**Fig. 1b**). This analysis reveals that the larger the number of NPs, the greater the cellular uptake. It observes that the increase of the nanofection load - ΔMFI units- is doubled when the fluorescent NPs per cell is doubled (**Fig. 1b**). This feature allows controlling nanofection loads which is crucial to determine the labelling incubation times. As expected, the uptake capability -nanofection load- of the different cell lines differ between them so these times need to be adjusted for each cell type. To corroborate that fluorescence detected by flow cytometry was generated by NPs located inside cells rather than NPs absorbed on the cell membrane, a confocal microscopy analysis was carry out. Confocal images for three orthogonal axes of the nanoparticle uptake are shown in **Supplementary Fig. 2**. It was observed the intracellular location of these NPs.

To further optimize the nanofection protocol, we performed a deeper study of the nanofection behaviour in a time course assay to determine the influence of the incubation time. We analyzed the nanofection percentage and nanofection load at different established time points (10, 20, 30, 40, 50, 60 and 75 minutes) using the ratio 1:25000 (cell:fluorescent NPs) (**Fig. 1c,d**). The ΔMFI analysis shows that the phenomenon of doubling nanofection load also occurs when the time is doubled (**Fig. 1c**). On the other hand, after 30 minutes of incubation, the 100% of all adherent cell lines were nanofected (**Supplementary Fig. 3**). The proportional increase of the nanofection load relative to the incubation time confirms the possibility to control the specific value of ΔMFI units obtained also changing the incubation time.

Once this method was optimised with adherent cells, human erythroleukemic cell line K562 was used as suspension cell model in order to evaluate the options of the method. Results

obtained with K562 presented the same features as when using adherent cells in terms of nanofection load and percentage (**Fig. 1d**). Furthermore, to verify the feasibility of this method, suspension hard-to-transfect cells, such as lymphoma cell lines Jurkat and Raji, were evaluated. Proportional increase of the nanofection load related to incubation time was also shown (**Fig. 1d**). In both cases, the best ratio cell:fluorescent NPs was 1:25000 and optimum incubation time was 30 minutes.

An important aspect to keep in mind when developing methods for proliferation monitoring is their potential cytotoxicity. The cell cycle was evaluated by flow cytometry following propidium iodine staining. Evidence of cell dead was not observed in any cell line (**Fig. 1e**). None of them presented a significant increment in the subG1 population after NPs treatment, showing any toxic effect. Instead, perfect matches between cell cycle profile of untreated and nanofected cells were observed after seven days of incubation. There is not a significant difference between the percentage of cells in each phase of the cell cycle in any condition ($p < 0.05$) (**Fig. 1d** and **Supplementary Fig. 4a**). Additionally, a long-term tetrazolium-based toxicity assay to assess mitochondrial function was performed in NP treated cells compared to untreated cells using two different NP:cell ratios. Same proliferation level was maintained in all conditions tested and no substantial metabolic changes due to NP treatment were observed (**Supplementary Fig. 4b**). These results suggest that nanofection load can be controlled in a robust manner through concentration and incubation time and cell-NP interaction does not adversely affect cell viability.

Predictive calculation of optimum range of nanofection

In this study, Δ MFI analysis revealed that median of fluorescence intensity decreases over time, and importantly this empirical observation was evident in all cell lines tested.

Surprisingly, this fluorescence decay was differed dependent on cell line. These data supported possibility of use doubling time as a key parameter for cellular-based assays using NPs in order to optimize cellular uptake. Previous simulations predicts an exponential decay of the average number of nanoparticles per cell with a decay constant given by where is the cell population doubling time [19]. Therefore, we propose a mathematic approximation to predict the correct NPs amount to be able to monitor cell proliferation. Accordingly, previous optimization assay will not be required for using this method with new cell lines. As mentioned above, time-point assay can be determined by calculating the nanofection load values together with population doubling time. Cells can theoretically be monitored until complete dilution of fluorescents NPs, i.e. when ΔMFI decrease to 1 unit. This end point is reached when median fluorescence intensity of nanofected cells is equal to median fluorescence intensity of non-nanofected cells (background). However, this fluorescent NPs dilution is also influenced by population doubling times of cell lines. In the division process of a nanofected cell, a parent-progeny transfer of NPs is produced. After each division, the nanofection load halves into daughter cells reducing fluorescence intensities -nanofection load- to half. This results in an exponential decay of the fluorescence division after division. This force us to consider the ratio “nanofection load: population doubling times” for long-term assay.

With this purpose, we calculate population doubling times of adherent and suspension cell lines. We observed that considering the number of cell divisions of each cell line in specific period of time, we can estimate the optimal range of nanofection load - ΔMFI units- that we need in order to monitor cells during that period of time. Applying the general formula that is used for exponential growth and decay formula we know the optimal ΔMFI value require at the start of the experiment:

$$Y=Y_0K^X$$

Y = Δ MFI units at the end of the experiment (equal to 1).

Y_0 = Δ MFI units at the start of the experiment.

K = Reduction rate constant (equal to 0.5; after each division the Δ MFI units are reduced to the half).

X = Number of divisions of each cell line in an specific period (days)

By applying this formula, an estimation of the initial range of nanofection load can be calculated. We applied that formula to our experimental data with MDA-MB-231, MDA-MB-468 and MCF-7 cell lines. The doubling time is respectively 38, 40 and 43 hours. Typical monitoring proliferation assays take 7 days. During this time, MDA-MB-231 cells divide 4.4 times, MDA-MB-468 cells 4.2 times, and MCF7 3.9 times. In contrast, suspension hard-to-transfect cells, Jurkat and Raji, proliferate faster. In consequence their doubling time is lower (23 and 25 hours respectively) and their division ratio in 7 days higher (7.30 and 6.72 times). In particular, using this predictive approach we found that to monitor MDA-MB-231, MBA-MB-468 and MCF7 cells, an initial nanofection load of around 21.4, 18.4 and 15 Δ MFI units, respectively, would be required. In the case of suspension hard-to-transfect cells, which have greater division ratio, higher Δ MFI units would be required (158.1 for Jurkat cells and 105.4 for Raji cells) (**Supplementary Table 2**). Remarkably, using the ratio 1:25000 (cell:fluorescent NPs), these Δ MFI values were obtained for all cell lines with a 30 minutes incubation time (**Fig. 1c and Supplementary Fig. 5**). These results confirm the predictive value of this mathematic approach. Using this exponential decay formula, an estimation of a range of fluorescence intensity initial can be successfully calculated.

Validation of this method

Once all parameters were efficiently established (concentration and time of incubation together with doubling time), we tested this novel method for tracking cell proliferation. For this purpose, a long-term assay of cellular monitoring was performed. Adherent and hard-to-transfect suspension cells were nanofected (25,000 NPs per cell, 30 minutes). Following nanofection, fluorescent NPs were distributed to daughter cells resulting in a progressive halving of the progeny fluorescence (**Fig. 2a, top panel**). This reduction of fluorescence intensity was quantified by flow cytometry. In order to monitor cell populations, harvesting was done at different time points (every 24 hours). Firstly, percentage and load of nanofection were analysed at initial time (Day 0) (**Supplementary Fig. 6**). Flow cytometry plots are shown in **Figure 2b-d**. During the progression of the assay, the reduction of nanofection load was clearly observed (**Fig. 2b**). Δ MFI was near 1 at day 7, validating the use of exponential decay formula to optimize the nanofection parameters (**Fig. 2c-d**).

To reinforce the feasibility of this method, an assay to monitor the reduction of cell proliferation was set up. For this purpose, cell proliferation was reduced by treatment with Mitomycin C (MCC), a cytotoxic drug that induces cell cycle arrest in G2/M phase as consequence of DNA damage. In a first stage, the cytotoxic effect of this drug was determined by flow cytometry analysis using propidium iodide staining (**Supplementary Fig. 7**). The arrest in G2/M impedes that cells initiate mitosis, producing a stop of cell division. According to this, cells do not proliferate after MMC treatment; therefore, if the cells are nanofected, they should maintain the nanofection load after MMC treatment (**Fig. 2a, bottom panel**). With this purpose we monitored nanofected cells after MMC treatment. Following nanofection step, nanofected cells were treated with MCC and cell proliferation was monitored for 7 days. Cells samples were fixed at different time points and analyzed by flow cytometry. **Fig. 2e** shows the results of analysis of cell arrest based on this method. As

expected, untreated cells suffered a complete loss of the fluorescence at day 7, a reduction which was progressive day by day according to an exponential decay. However, the fluorescence intensity of MMC treated cells remained at day 7; there was not a reduction of fluorescence intensity compared to day 0 meaning that proliferation had stopped. This data confirms the feasibility of the presented method. In the particular case of suspension cells (Raji and Jurkat), an unexpected reduction of the fluorescence is observed in treated cells with MCC. This can be the result of high doubling times of these cells and delayed effect of MCC. This was confirmed by the time course monitoring of the MCC effect in these cells. At Day 4 (96 h), a significant difference was observed and, consequently, the effect of this drug can be studied after 96 hours, time at which MMC treatment was totally effective (**Supplementary Fig. 7 and 8**).

Monitoring primary lymphocytes proliferation.

Success of adaptive immune system depends on lymphocyte proliferation. The ability to accurately predict proliferative behaviour of lymphocytes has important implications for human health research. Standard methods to measure cell proliferation based on fluorescent dyes are particularly toxic in this kind of cell [20]. Therefore, alternative methods which were not cytotoxic are required. This fact led us to assess our method for measuring lymphocyte proliferation. For this purpose, in the first stage, the nanoparticle uptake capacity of isolated human primary PBMC (monocyte depleted-peripheral blood mononuclear cells) was tested. PBMCs were incubated with Cy5-NP and the efficiency of NPs internalization was analysed by confocal fluorescence microscopy and flow cytometry. A confocal fluorescence microscopy confirmed the nanoparticles intracellular localization just after nanofection (**Fig. 3a**). In parallel, a flow cytometry analysis was performed. It was found that our nanofection

parameters managed to reach 90% nanofected population (**Fig. 3b-c**). Nanofected population percentages can increase by varying incubation time and number of added NPs per cell.

Once the efficiency of cellular labelling using NPs was proven, and in order to assess our method for measuring lymphocyte proliferation, the PBMCs were nanofected and stimulated to induce their proliferation. The analysis was performed following 7 days after PHA-induced proliferation. The nanofection load practically disappeared due to the induced lymphocyte proliferation. In fact, fluorescence intensity decrease was gradually observed over days due to Cy5-NPs dilution between the progeny as consequence of induced cell division (**Fig. 3d**). At day 7, the histogram plot shows a slight peak of positive population which corresponds to non-proliferative cells, something expected due to the fact that, following stimulation step, not all cell population participate in the division (**Supplementary Fig. 9a**). Overall, the data confirms the absence of deleterious effect of Cy5-NPs in the PBMC viability.

In order to confirm our data, comparison of our nanotechnology-based method with the wide accessible and most popular method to measure lymphocyte proliferation (intracellular fluorescent dye, CFSE) was performed [20–22]. We confirmed that substantial cell proliferation was evident only after 3 days of culture as common feature of T and B cells responding to mitogens and specific antigens. CFSE dye dilution was observed in the tested successive time points (**Supplementary Fig. 9b**). The data validates the applicability of the method to measure lymphocyte proliferation.

Discussion

A good candidate for tracking cell proliferation using fluorescence techniques must have certain specific characteristics such as (1) have bright fluorescent features, (2) being readily taken up by cells, (3) being evenly distributed within progeny cells, (4) displaying slow

dilution rates and (5) being non cytotoxic [5,6]. The main advantages of using nanoparticles rather than classical approaches are that: a) they are more stable than fluorophore in solution [15], b) the fluorescence signal does not decrease rapidly during the first 24 hours after labelling, then zeroth generation can be set up using cell sample from day 0 [23] and c) they can be used to track a broad range of cellular types without any cytotoxic effect allowing its application in many biological/biomedical research fields [18]. To the best of our knowledge, cell clone formation assay based on nanotechnology imaging technique by using Quantum dot, has been the only reported to date to study the proliferative features [19]. In this work, we have evaluated a nanotechnology based method to monitor cell proliferation by analysing these properties that a good candidate for tracking cell proliferation must offer. To do so, we have used 200 nm PS NPs labelled with a near infra-red fluorophore (Cy5) to be able to evaluate these nanodevices by fluorescent techniques. However, other fluorophores of different nature could be used instead of Cy5. On the other hand, the role of surface charge of polystyrene nanoparticles on cellular uptake is still controversial. Carboxylated PS-NPs were ingested to a higher degree by alveolar type I cells [24], whereas preferential uptake of cationic PS-NPs has been observed in Madin Darby canine kidney cells [25]. However, as previously reported, we have found that the presence of free amino groups on the NP surfaces is essential to allow an efficient cell labelling [26]. Based on these findings, we have designed a nanodevice that contain a defined amount of free amino groups to ensure an efficient cellular uptake with high rate, allowing an effective internalisation and consequently an efficient cell labelling to be able to monitor cell proliferation in long term assays. Nanoparticles do not present cytotoxic effects in the cell lines tested, being this fact an essential characteristic therefore making them an ideal method to monitor cell proliferation.

Interestingly this method could be implemented using nanoparticles of different nature as far as they do not affect cell viability.

We demonstrated that these nanodevices are readily taken up by the cells, and they present slow dilution and non-effect on the cell function. On the other hand, it is already accepted NPs are split between daughter cells when the parent cell divides [26]. Whether their distribution is equal during cell division still remains controversial. Future work could be focused on the determination of nanoparticle dose in a statistical framework by imaging microscopy using our fluorescent NPs as nanotracker. Nevertheless our results support the efficiency of this method. We have proven that the measurement of Δ MFI is a reliable parameter to monitoring the linearity over time, regardless of eventual asymmetry. MFI is more robust than mean values and heterogeneity from cell to cell is considered [27]. In the other hand, because of it is known that used hard-to-transfect cells are specialized in avoid uptake, we considered NPs could be remained associated to membrane cells. Hence, we have discriminated between NPs absorbed on the cell membrane or NPs internalized inside the cell, providing solid correlation between fluorescent signal observed and proliferation. Regarding cell cycle stage, differences in cellular nanoparticle uptake levels have been previously reported [26]. However, these differences are not evident during the first 5 h of exposure. Consequently, as shorter incubation time is needed in this methodology (only 30 minutes), there is not influence of cell cycle phase in the uptake of these nanoparticles.

Changing parameters such as concentration and time of incubation, the nanofection load can be controlled in a robust manner in order to reach an optimum MFI initial value. We have demonstrated that 25000 NPs and short period of nanofection time (30 min) were adequate to achieve satisfactory MFI values to monitor cell proliferation during 7 days. Likewise, same MFI values could be accomplished applying lower number NPs added per cell and higher

incubation time. Even so, the highest ratio that has been used for this method is 50000 NPs added per cell. However, in previous work, higher concentration without observing any cytotoxic effect was successfully used [18]. Accordingly, the use of this method allows the fluorescence labelling of cells with high intensity and reproducibility (very low variance). In fact, this is a method of broad application as the unique cell requirement to be able to monitor cell proliferation using this nanotracker is efficient internalisation [27].

Because of these nanoparticles are not exocytosed by cells as previously was reported [28], make them an excellent candidate for cell proliferation monitoring, as the only thing diluting the signal is the division of the cell in two daughter cells. The fact that these nanoparticles are not toxic for any cell lines tested so far is also of remarkably importance as they do not affect the cell function. Indeed, lymphocyte viability after NP internalization and PHA-induced proliferation has been demonstrated. This observation suggests that NPs are non-toxic in systems cultured with less serum (such as PMBC), as it was previously reported [29], allowing this methodology to be considered in different physiological media.

It is important to mention that, due to the fact that these fluorescent nanoparticles are labelled with a far red dye, then a multicolour flow cytometry assay can be set up as before reported [19], but without interference of the nanodevices with other fluorophores labelled reagents (such as fluorescein-labelled antibodies), therefore avoiding cell autofluorescence.

Finally, we have proposed the use of NP-based cell tracker as a potential tool to monitor lymphocyte proliferation minimizing toxicity, and thereby being required lower initial lymphocytes number to assess. Our methodology has also proven beneficial

for *in vitro* studies as possible alternative for the current cell tracking using fluorescent protein and membrane dyes [21,30].

Conclusion

The method has been validated in several adherent cells and also in suspension cells including hard-to-transfect cells. Even more interesting is the fact that monitoring of cell proliferation of lymphocytes has been successfully achieved (so far the only efficient method to do this lymphocytes monitoring is CFSE staining). The method was also validated in a cell-based assay which determined the induced cellular arrest through MMC effect, showing the power of the method for long-term cellular assays. Furthermore, this new method does not alter the cell cycle hence presenting no cytotoxic effects. This fact corresponds to previously findings where NPs were never found associated with any components of the mitotic apparatus and no abnormal cell division was detected after their internalization [31].

In conclusion, an alternative method to monitor cell proliferation, which can be used with a large variety of cell lines for long term cellular assays, is presented. We propose the use of nanoparticles as an accessible tool to track cell proliferation for any laboratory which could access nanotechnology-based approaches.

Summary points

- An efficient nanotechnology fluorescence based method to track cell proliferation has been validated.
- Polystyrene nanoparticles (NPs) has been successfully synthesised, PEGylated, funtionalized, fluorescently labelled and characterized.

- These NPs has been assessed for in vitro long-term monitoring of cell proliferation in several adherent and suspension cells including hard-to-transfect cells and isolated human primary lymphocytes..
- These nanoparticles are stable and not toxic for any cell lines tested so far.

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Financial & competing interests disclosure

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Reference annotations

Author contributions

MVCC synthesised and characterised amino functionalised polystyrene nanoparticles. MVCC and MPRB prepared fluorescent nanoparticles. PAM, JDUB and MTVG carried out biological experiments. PAM and JDUB analyzed raw data and performed statistical analysis. JDUB, RSM and JJDM contributed to design biological experiments. PAM, JDUB, JJDM and RMSM interpreted the biological data and wrote the paper. RMSM directed the work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

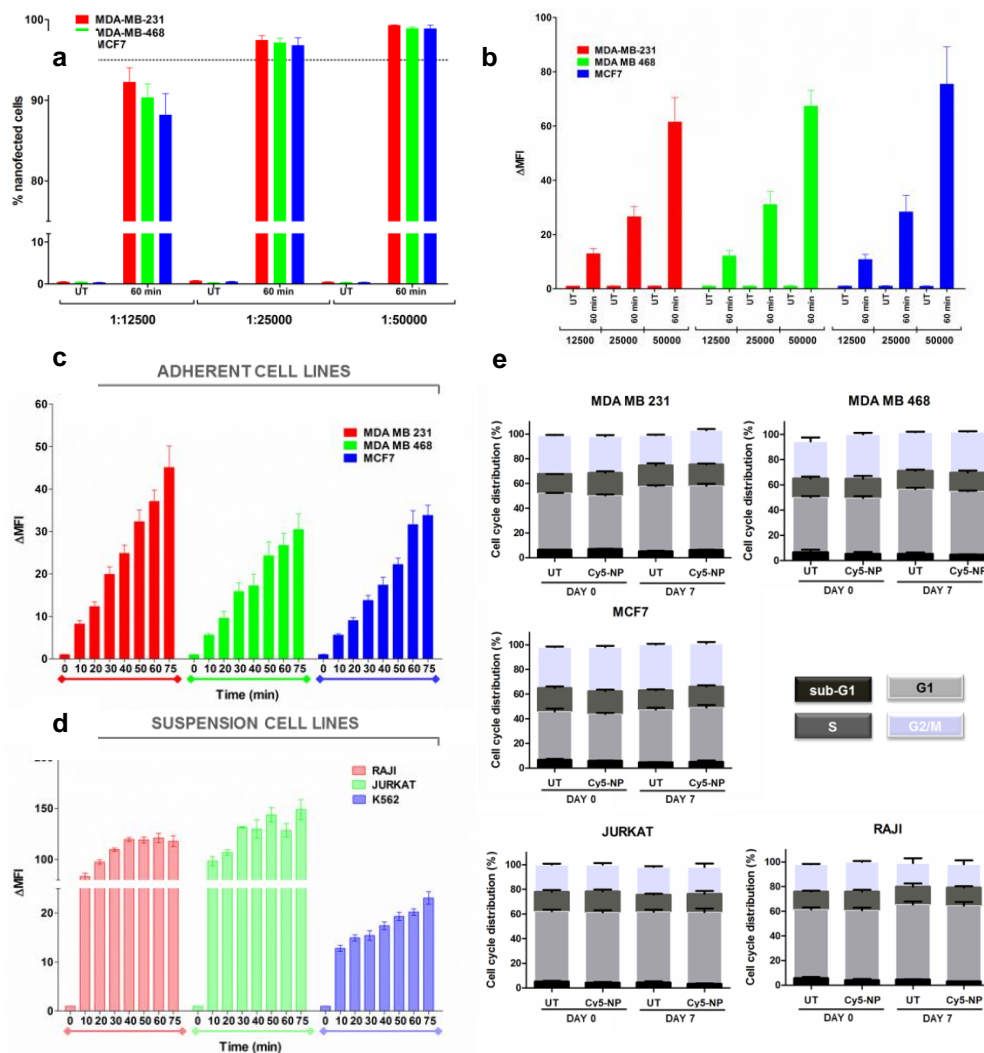


Figure 1. Optimisation of the nanofection method in the different tested cell lines. (a) Analysis of Cy5-NPs cellular uptakes by MDA-MB-231 (red), MDA-MB-468 (green) and MCF7 (blue). Cy5-NPs, at different ratios per cell, were incubated and analysed by flow cytometry and percentage of cells containing Cy5-NPs is displayed. Saturation percentage is represented above dotted line. (b) Study of median of fluorescence intensity increments (Δ MFI) at different Cy5-NPs ratios per cell (12500, 25000 and 50000) compared to cells without NP-treatment. Values for cells incubated with Cy5-NPs for 60 minutes and untreated cells (UT) are shown. (c,d) Study of median of fluorescence intensity increments (Δ MFI) at different incubation time points. (c) Strong colours panel shows results for adherent cell line models and (d) light colours panel represents suspension cell line models. (e) Cell cycle distribution in cell lines before and after long term nanoparticle incubation. The percentages of cells in different phases of the cell cycle were determined from the histograms by flow cytometry. Adherent cell lines (upper and middle panels) and suspension hard to transfect cell lines (lower panels) were tested. Graphs summarize data from initial (Day 0) and final (Day 7) experimental time points. Bars represent mean \pm SEM of results from 3 independent experiments with duplicated points. UT: untreated cells. Cy5-NPs: fluorescence nanoparticles.

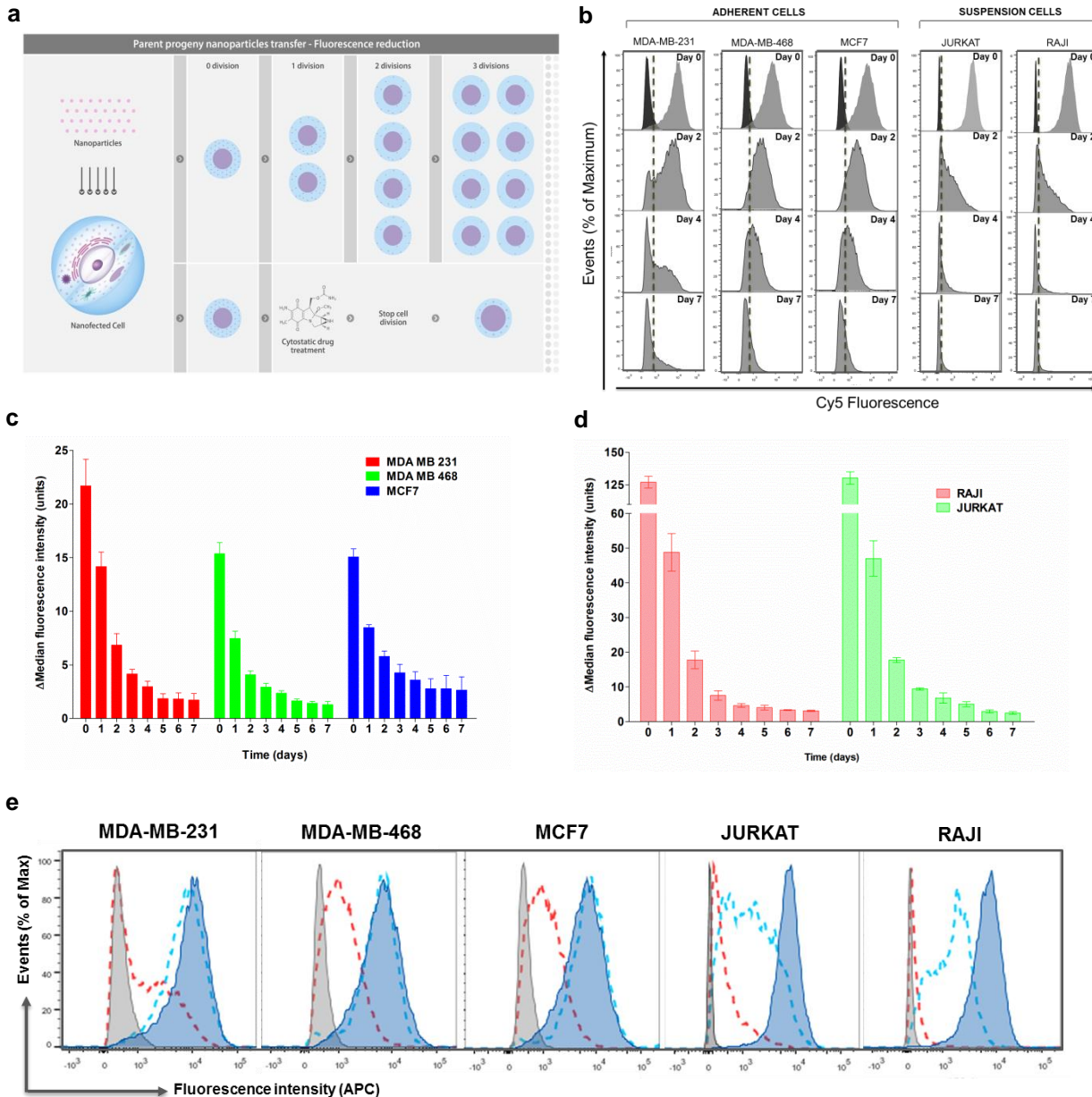


Figure 2. Validation of the nanofection method in order to monitor cell proliferation. (a) Schematic representation of cell labelling by nanofection and the nanoparticle distribution into daughter cells, in a theoretically equal distribution, resulting in a progressive halving of the progeny fluorescence. **(b)** Representative histogram plots of untreated cells (dark peak) compared to Cy5-NPs treated cells (light peaks) at day 0, 2, 4 and 7. Reduction of the nanofection load was observed over the days. **(c,d)** Flow cytometry was performed every 24 hours and increment of median fluorescence intensity measured (Δ MFI) is represented for (c) adherent cell lines and (d) suspension hard to transfect cell lines, **(e)** Flow cytometry analysis of untreated and nanofected cell lines after Mitomycin C treatment. Open dashed histograms represent 7 days incubation time point, whereas the filled histograms depict initial experimental time point. Each panel shows a comparison between untreated cells (grey filled histogram) and cells treated with Cy5-NP (25000 added per cell) for 30 min (blue filled

histogram). The graphs show that fluorescence intensity decrease after 7 days of incubation due to cell division (dashed red histogram) compared to initial fluorescence. In contrast, in cells which cell cycle is arrested, nanoparticles have not been split and fluorescence intensity is maintained over time (dashed blue histogram). Each histogram is representative of at least 3 experiments and shows percentage of maximum (Y axis) versus log fluorescence intensity (X axis) for 10.000 viable cells.

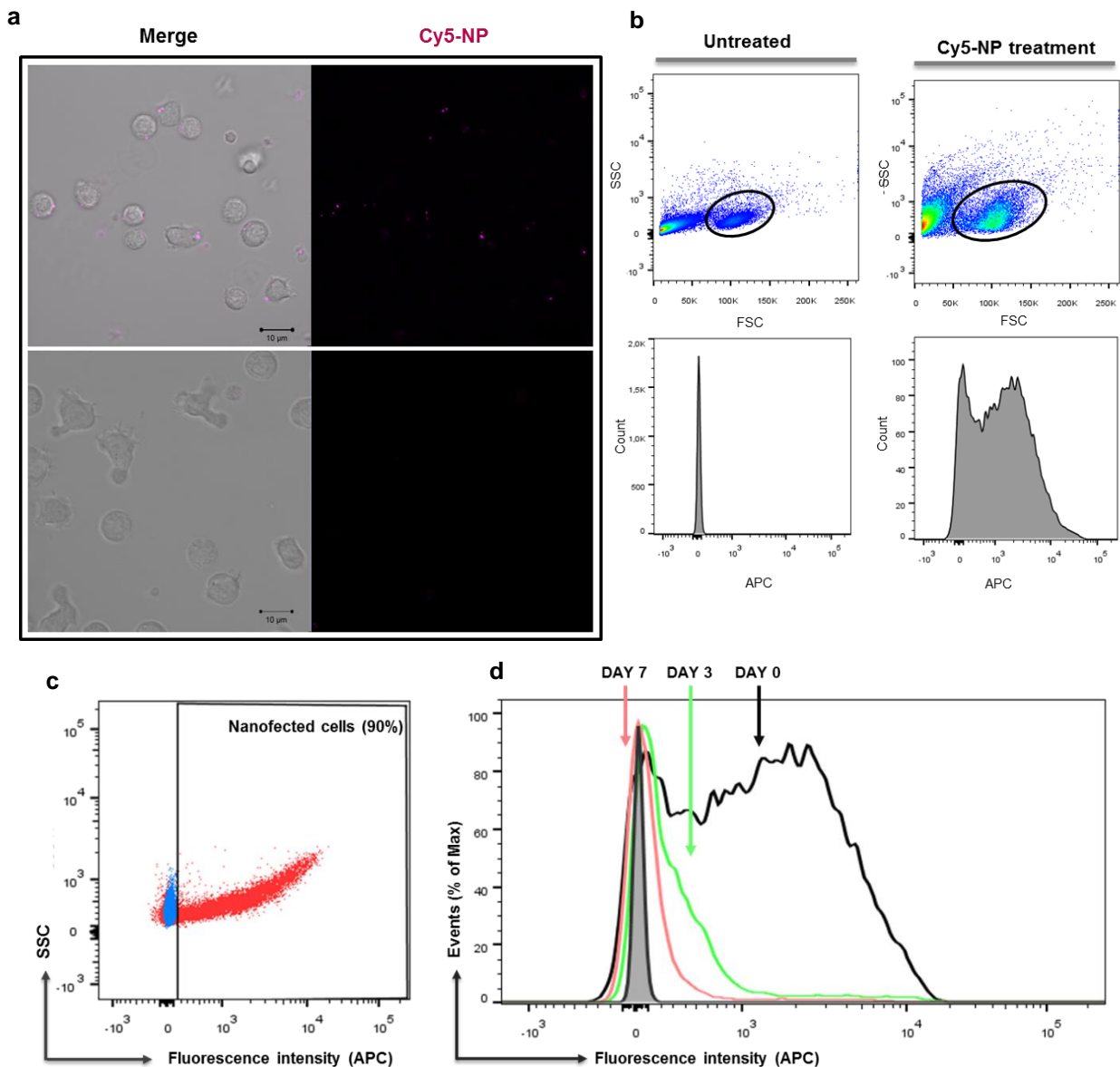
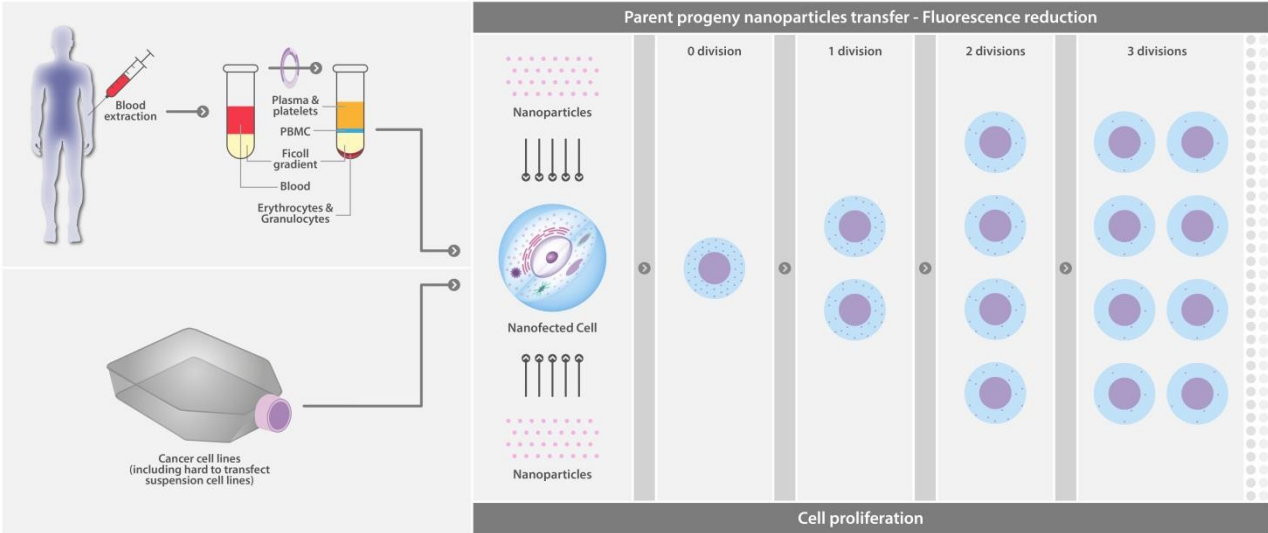


Figure 3. Demonstration of Cy5 labelling lymphocytes and proliferation detection and monitoring of proliferating lymphocytes. (a) Confocal fluorescence microscopy to confirm Cy5-NPs internalization by freshly isolated lymphocytes (top panel) and demonstration of Cy5-NPs dilution after 7 days caused by proliferation of lymphocytes stimulated (bottom panel). Left panel: Merge, composition of the two recorded channels (DIC–Differential interference contrast– and red, Cy5-NPs). Right panel: red channel, Cy5-NPs. Scale bar, 10 μ m. (b) Analysis of the specific lymphocyte populations depending on treatment provided. Freshly isolated lymphocytes were subjected to the same procedure without nanoparticles (represented in left panel) and they were considered as untreated cells; right panel shows lymphocytes after 30 min Cy5-NP incubation. The results show lymphocytes population is not affected when Cy5-NPs were used. (c) Nanofected population (red) after 30 minutes of Cy5-NPs incubation versus untreated cells (blue) represented by dot plots. (d) Fluorescence

intensity dilution over days in PHA-induced lymphocytes (open histograms) versus untreated cells (filled grey histogram). Results are from a representative experiment.



Graphical abstract.

Supplementary Information

Tracking cell proliferation using a nanotechnology based approach

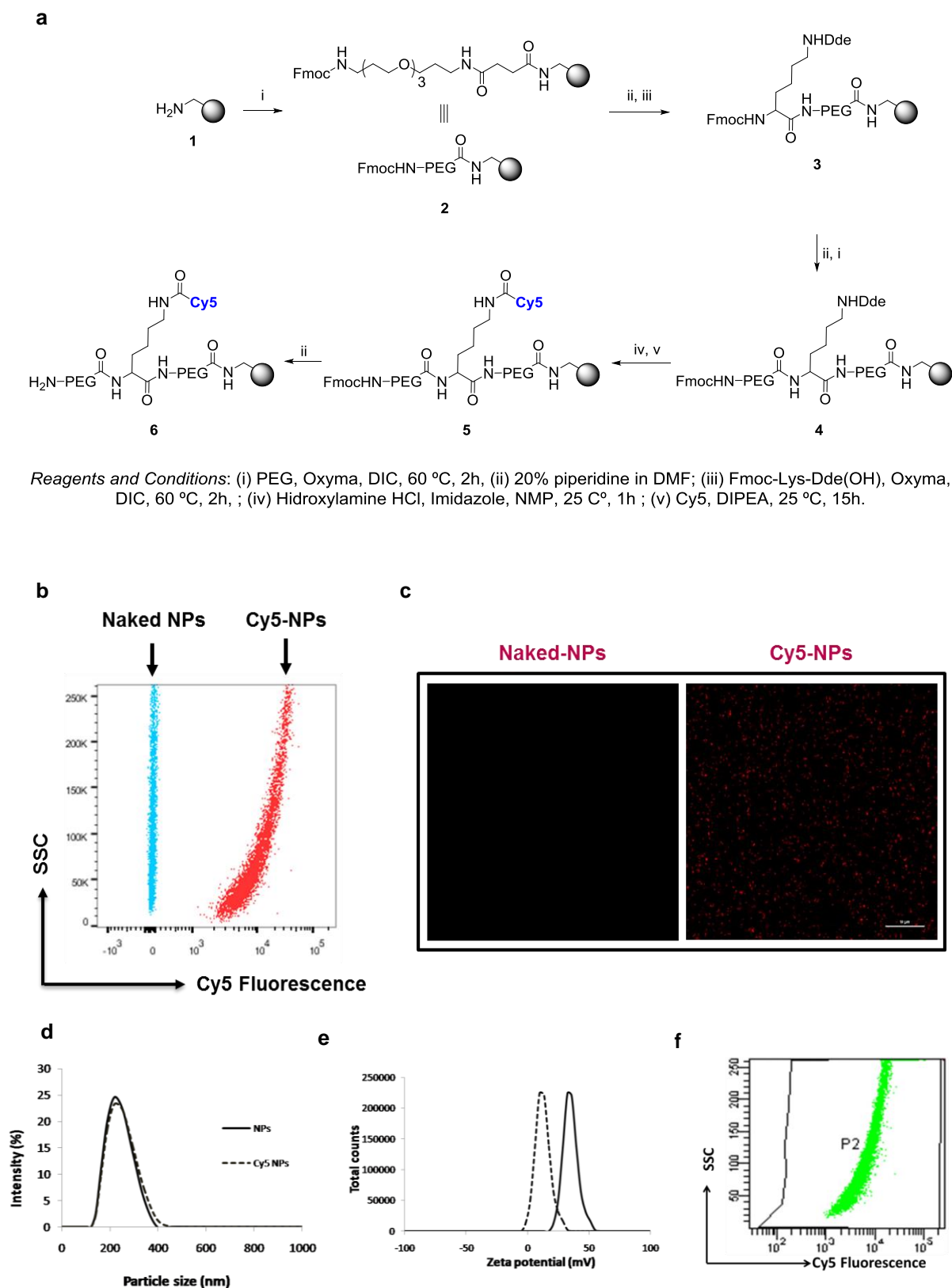
Patricia Altea-Manzano,^{a,c} Juan Diego Unciti-Broceta,^{c,*} M^a Victoria Cano-Cortés,^{a,b} María Paz Ruiz-Blas,^{a,b} M^a Teresa Valero-Griñán,^{a,b} Juan Jose Díaz-Mochón^{a,b} and Rosario M^a Sanchez-Martin^{a,b,*}

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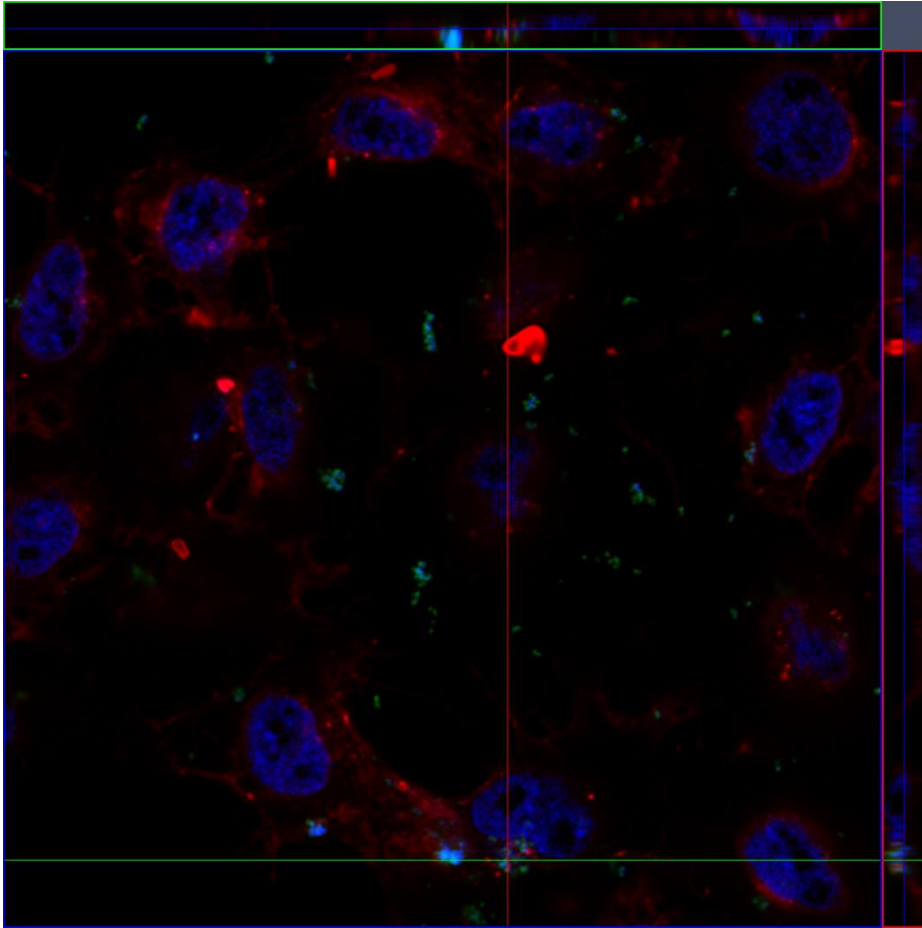
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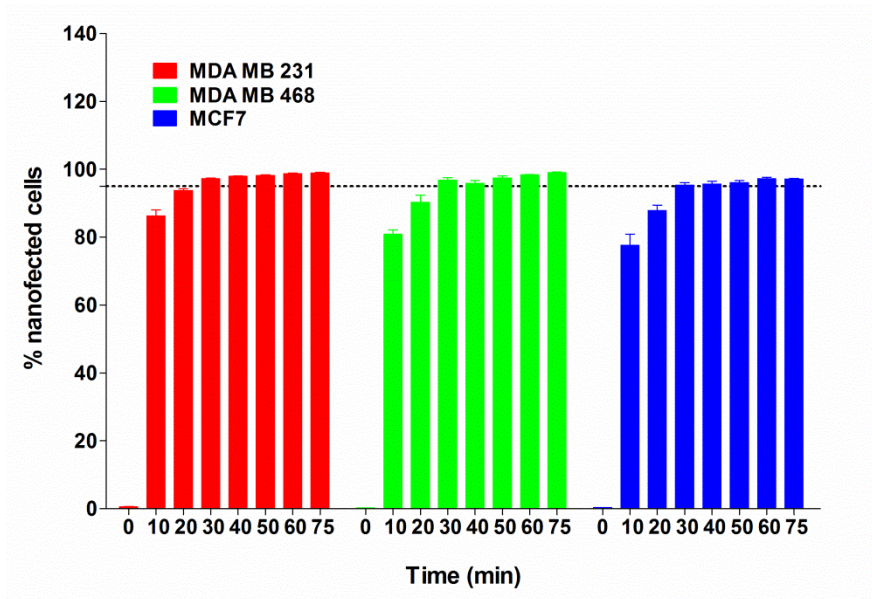


Supplementary Fig. 1. Fluorescence nanoparticles synthesis and characterisation. (a) Schematic synthesis of fluorescence NPs. Abbreviations: PS, polystyrene; PEG, Fmoc-1-amino-4,7,10-trioxa-13-tridecamine succinic acid, polyethylene glycol; NP, nanoparticle. **(b)** Representative overlay dot plot obtained after flow cytometry analysis of naked NPs (blue)

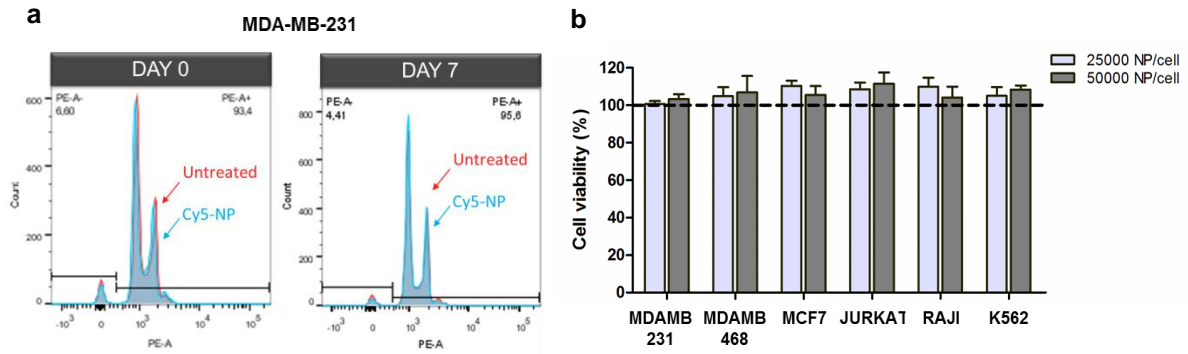
and Cy5-NPs (Red). **(c)** Confocal fluorescence microscopy of naked NPs and Cy5-NPs. DIC, Differential Interference Contrast. Scale bar, 10 μm . **(d)** Particle size distribution (nm) and **(e)** Zeta potential values of amino NPs (NPs) and Fluorescent labelled amino NPs (Cy5 NPs). **(f)** Flow cytometry analysis of Cy5-NPs suspended in culture media supplemented with serum after 7 days incubated at 37°C. SSC, side scatter.



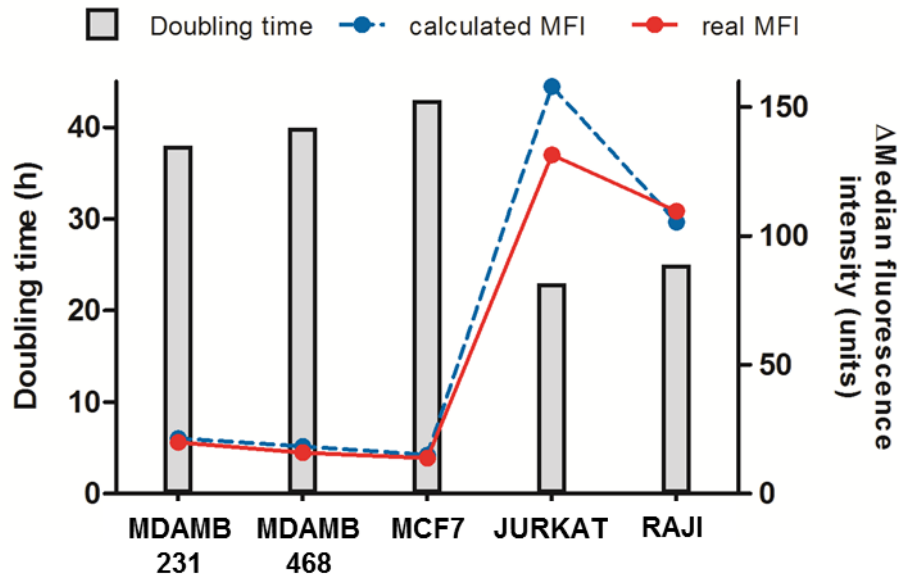
Supplementary Fig. 2. Representative confocal section image and orthogonal projections of MCF7 to analyze Cy5-NPs cellular uptake. Optical sections are displayed in three orthogonal projections [xy-projections (main panel), xz-projections (top panel), and yz-projections (right panel)] to distinguish between extracellular and internalized nanoparticles. The yellow lines indicate the positions of the xz and yz planes. Channels (red, PKH26 Red Fluorescent Cell Linker -membrane stained-; blue, DAPI -nuclei-; and green, Cy5-NPs).



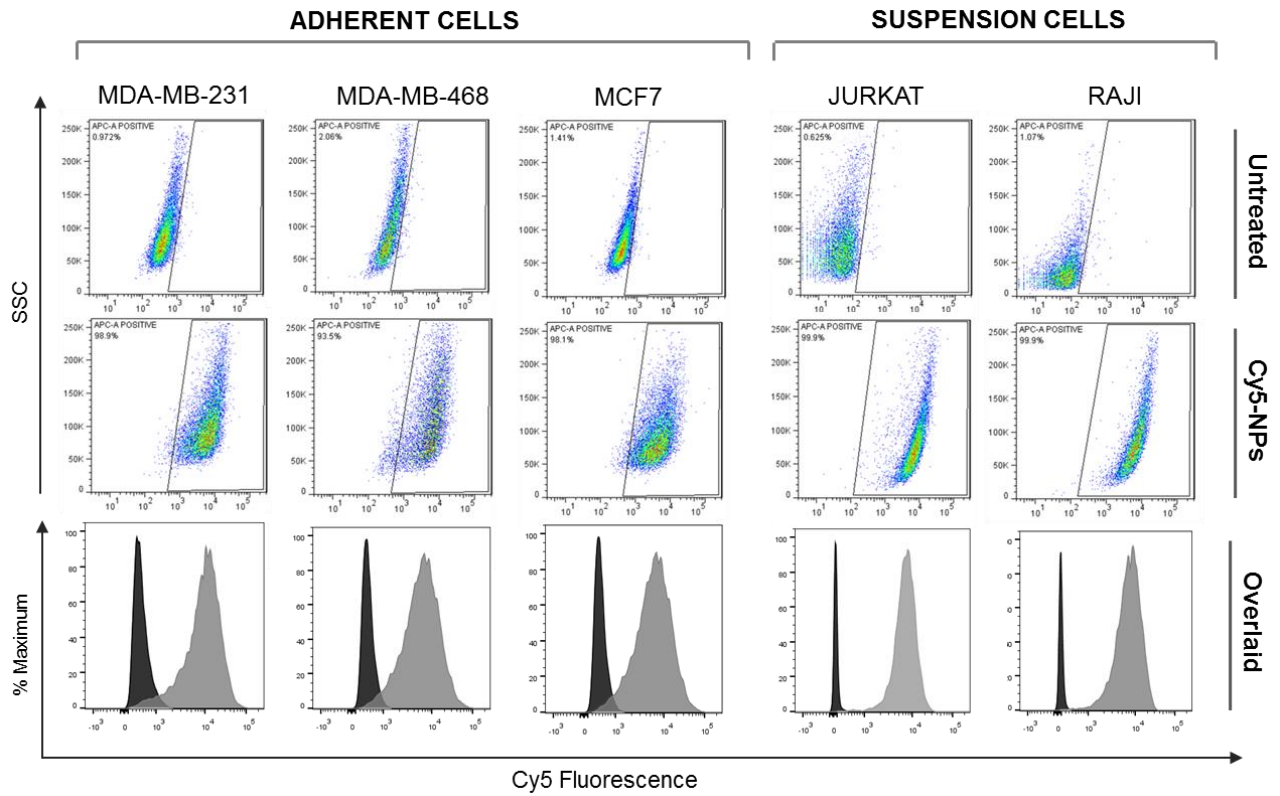
Supplementary Fig. 3. Analysis fluorescence-NPs cellular uptakes by adherent cell lines. Cy5-NPs, at different time points, were incubated with MDA-MB -231 (Red), MDA-MB-468 (Green) and MCF7 (blue) cell lines and analyzed by flow cytometry. Percentage of cells containing Cy5-NPs versus cell to Cy5-NPs ratio is displayed in a bar representation to compare cellular uptake. >95% of Cy5 positive cells was reached at 30 minutes from 1:25000 ratio (cell: Cy5-NPs), represented by dotted line. Results are expressed as mean \pm S.E.M.



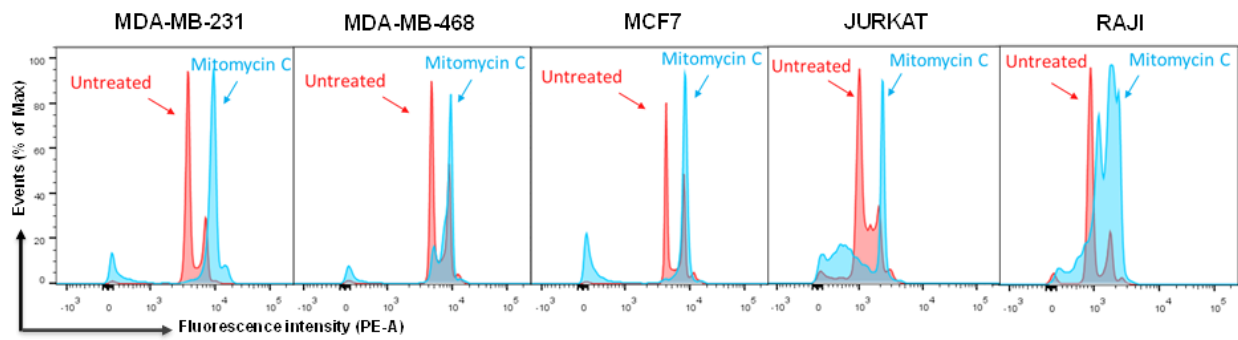
Supplementary Fig. 4. Effect of NP treatment on cell viability and proliferation during time course experiment. (a) Ploidy histogram of the relative DNA content was determined in cells incubated with NPs (blue) compared with untreated cells (red). Cell cycle profiles showed identical viability in cells with and without NP treatment. Figure shows a representative adherent cell line (MDA-MB-231). Identical effect was observed in the rest of the cell lines tested. **(b)** Proliferation of NP treated cells (1:2500 and 1:5000 NPs added per cell) referred to untreated cells (100%) measured in adherent and suspension cell lines.



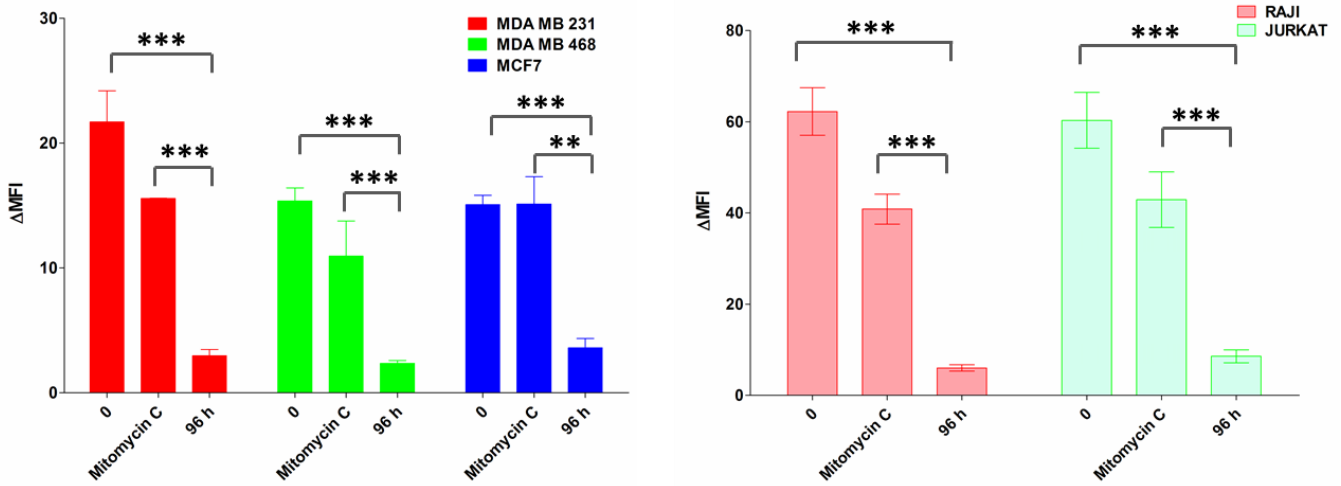
Supplementary Fig. 5. Higher values of Δ MFI are predicted when cell doubling time is lower by using the exponential decay formula. No differences between the predicted values of Δ MFI (dashed blue line) and those empirically obtained (red line) were observed.



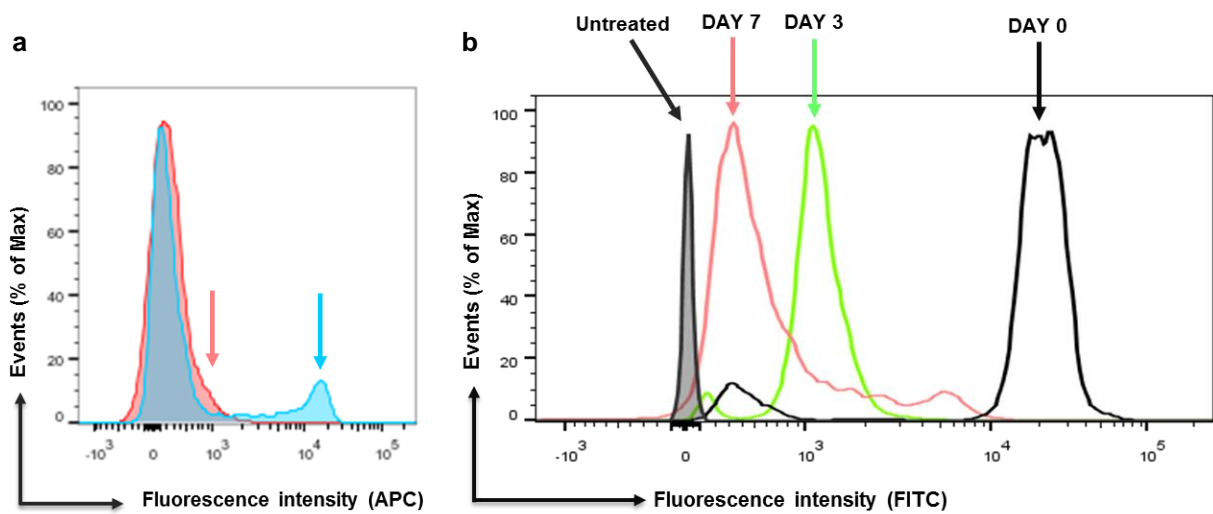
Supplementary Fig. 6. Representative flow cytometry dot plots obtained with different cell lines 30 minutes incubated with 1:25000 cell to Cy5-NPs ratio. Representative Dot plots of untreated (top panels) and Cy5-NPs treated cells (25000 NPs, 30 minutes) (middle panels) are showed. Histogram overlaid of untreated cells and Cy5-NPs treated cells are showed in lower panels. SSC: side scatter, cellular complexity.



Supplementary Fig. 7. Cell cycle arrest in G2/M phase as consequence of Mitomycin C treatment. DNA histograms generated by propidium iodide staining and flow cytometry analysis for adherent and suspension hard to transfect tested cell lines. The DNA histograms show a distribution comparison of cell populations in each phase of the cell cycle between untreated cells (red histogram) and cells treated with Mitomycin C for 96h (blue histogram). . Each histogram is representative of at least 3 experiments



Supplementary Fig. 8. Study of fluorescence differences between untreated cells and Mitomycin C treated cells in nanofected cell lines. Increment of median fluorescence intensity (Δ MFI) versus Cy5-NPs treated cells at initial proliferation time-point (0), after 4 days of proliferation (96h) and after 4 days of MMC treatment. Results are expressed as mean \pm S.E.M. Statistical significance was determined by Bonferroni's multiple comparison test between the different treatments of each cell lines.



Supplementary Fig. 9. (a) Histogram plot comparing both PHA-induced nanofected (blue) and non nanofected (unlabelled) cells (red) after 7 days. Higher blue peak represents stimulated nanofected cells which have diluted their fluorescence. Blue arrow shows a slight peak corresponding to fluorescence measurement of non-dividing lymphocytes. Red arrow highlights unlabeled population, which provides a measurement of the autofluorescence of an activated lymphocyte population. **(b)** CFSE labelling dilution (FITC fluorescence) over days in PHA-induced lymphocytes (open histograms) versus untreated cells (filled grey histogram).

Time (days) 37°C	Size (nm)*	PDI	Zeta potential (mV)
0	205.6	0.051	19.3
1	206.1	0.069	18.9
2	202.3	0.087	19.6
3	204.9	0.102	17.8
4	205.1	0.056	18.5
5	202.9	0.075	19.1
6	206.8	0.098	18.4
7	205.4	0.063	19.7

* Dynamic Light Scattering analysis

Supplementary Table 1. Study of stability of Cy5-NPs suspended in culture media supplemented with serum versus time (temperature 37°C). The *in vitro* stability studies show that, the Cy5-NPs were highly stable under the experimental conditions adopted. PDI: polydispersity index.

$$y = y_0 \times k^x \rightarrow 1 = y_0 \times 0.5^{\text{division number}}$$

Doubling time (hour)	Cell divisions in 7 days	0.5 ^{division number}	Y0 in MFI units	
48	3,5	0,09	11,3	
47	3,6	0,08	11,9	
46	3,7	0,08	12,6	
45	3,7	0,08	13,3	
44	3,8	0,07	14,1	
43	3,9	0,07	15,0	⇒ MCF7
42	4,0	0,06	16,0	
41	4,1	0,06	17,1	
40	4,2	0,05	18,4	⇒ MDA-MB-468
39	4,3	0,05	19,8	
38	4,4	0,05	21,4	⇒ MDA-MB-231
37	4,5	0,04	23,3	
36	4,7	0,04	25,4	
35	4,8	0,04	27,9	
34	4,9	0,03	30,7	
33	5,1	0,03	34,1	
32	5,3	0,03	38,1	
31	5,42	0,02	42,8	
30	5,60	0,02	48,5	
29	5,79	0,02	55,4	
8	6,0	0,02	64,0	
27	6,2	0,01	74,7	
26	6,5	0,01	88,1	
25	6,7	0,01	105,4	⇒ RAJI
24	7,0	0,01	128,0	
23	7,3	0,01	158,1	⇒ JURKAT
22	7,6	0,01	199,0	

Supplementary Table 2. Calculation of range of MFI units needed to 7 days proliferation monitoring experiment by exponential decay formula depending on cellular doubling time. Different doubling time values are showed in the first column. Median of fluorescence intensity, showed in the last column, is calculated based on exponential decay formula (on the top), regarding 7 days of the proliferation monitoring. Calculated doubling time for each cell line used in cell monitoring experiments is outlined. Therefore, considering the number of cell divisions of each cell line in that specific period of days, an estimated optimal range of nanofection load -ΔMFI units- needed was obtained.

Supplementary Material & Methods

Preparation of Fluorescent nanoparticles (NPs)

Amino-methyl cross-linked polystyrene 200 nm nanoparticles were coupled with Fmoc-1-amino-4,7,10-trioxa-13-tridecamine succinic acid ref using standard HOBt (1-hydroxybenzotriazole)/DIC (1,3-Diisopropylcarbodiimide) chemistry. PEGylated NPs were centrifuged (13000 rpm), washed three times with dimethylformamide (DMF) before deprotecting the Fmoc group with 20% piperidine in DMF with three consecutive treatments, 20 min each, at room temperature and shaken at 1400 rpm. Fmoc deprotected NPs were then washed three times with DMF and Fmoc-Lys(Dde-OH)ref was conjugated followed by Dde deprotection and Cy5 conjugation (near infra-red dye). For the coupling, deprotected PEGylated 200 nm NPs were resuspended in DMF with DIPEA and commercially available Cy5 NHS ester (Lumiprobe) was added. The coupling was performed overnight at room temperature and at 1400 rpm. Finally, NPs (fluorescent near infra-red polystyrene NPs, referred in the main text as Cy5-NPs) were Fmoc deprotected with 20 % piperidine in DMF (x3) and two times washed with DMF. Culture suitable NPs were obtained by washing steps with methanol, ethanol, 70 % ethanol and resuspension in sterile water. The stability of the Cy5-NPs incubated in culture media over a period of time of 7 days at 37°C was monitored using flow cytometry.

Determination of fluorescent NPs concentration (NPs per microliter) by spectrophotometric method.

Fluorescent NPs concentration (NPs per microliter) was determined by a spectrophotometric method as described previously by Unciti-Broceta JD et al. [18].

Fluorescent NPs uptake study by flow cytometry

Adherent cell lines were washed with phosphate buffered saline (PBS 1X), detached with trypsin/EDTA (0.25%, phenol red) (Gibco), counted and diluted with media to a final concentration of 10^5 cells per mL. 500 μ L of each cell line suspension were plated in 24 well plates (Nunc) and incubated for 18 h. Then, media was replaced with 500 μ L of fresh media mixture containing specific number of Fluorescent NPs. The cells were incubated with the fluorescent NPs at the established incubation times in a humidified incubator at 5% CO₂ and 37°C. As control were used naked NPs (at the specific ratio cell/NPs), and cells without nanoparticles treatment. Suspension cells were harvested, washed with PBS1X, counted and diluted with serum free media to a final concentration 1×10^6 cells per mL. 600 μ L of each cell line suspension were placed in 1.5 mL conical culture tube (Eppendorf). Then, the specific number of Fluorescent NPs was added and cells were incubated in a humidified incubator at 5% CO₂ and 37°C. There were studied the same ratios cell:fluorescent NPs and incubation times that in adherent cell lines, using the controls described above.

After incubation with Fluorescent NPs, for adherent cells, the media was aspirated and cells were washed with PBS 1X, and detached with Trypsin-EDTA (0.25%) at 37° C for 5 minutes; for suspension cells, the media was removed by centrifugation and once washed with PBS 1X. In the case of suspension hard-to-transfect cells, additional wash step adding a reducing agent (tris(2-carboxyethyl)phosphine, Fluorochem) was done in order to quench possible binding nanoparticles in the membrane extracellular side, avoiding fluorescence interference from unincorporated NPs. Then, each sample was fixed in 2% paraformaldehyde (PFA) at room temperature for 10 minutes and protected from the light. Samples were analyzed via flow cytometry with a FACSCanto II flow

cytometer (Becton Dickinson & Co., NJ, USA). Each experiment was done in duplicate per ratio and incubation time and repeated three times per cell line.

Fluorescent NPs uptake study by confocal microscopy

Cells were plated into glass poly-L-lysine-coated coverslips (Neuvitro) in 24 well plates and incubated for 12 h. Then, media was replaced with a fresh media mixture containing Cy5-NPs and cells without nanoparticles treatment were used as controls. Following 30 minutes of incubation with Cy5-NPs, the media was aspirated and cells were washed with PBS 1X, and fixed in 4% paraformaldehyde (PFA) at room temperature for 30 minutes. Fixed cells were washed with PBS 1X and coverslips were mounted with ProLong Gold antifade mountant with DAPI (Life technologies). Image acquisition using fixed cells was performed with Confocal Scanning Microscope Zeiss LSM 710 Axio Observer (Carl Zeiss, Jena, Germany). Images were acquired using Objective Plan-Apochromat 63x/1.40 Numerical Aperture OIL DIC M27 and Zen 2010 (Carl Zeiss, Jena, Germany). Images were processed Image J versión 1.49b software. Each experiment was done in triplicate and repeated two times per cell line.

Cell-Cycle Distribution Measurements

Cell cycle analysis by quantitation of DNA content was performed using propidium iodide. Cells were harvested, washed twice with PBS, and fixed with 70% cold ethanol. After overnight refrigeration at -20°C, cells were washed again with PBS and cell nuclei were stained for 15 min in the dark with 50 µg/mL propidium iodide containing 100 U/ml of ribonuclease A, and added into a flow cytometry tube. DNA content was measured by flow cytometry (BD FACS Canto II) and the percentages of

cells in the G0/G1, S and G2/M phases were determined from DNA content histograms using FlowJo software.

Nanoparticle cytotoxicity assay

Cells were properly disposed to nanoparticle incubation as described above, using 1:25000 and 1:50000 cell:NPs ratio and 30 minutes of nanofection. After that, untreated and nanofected cells were plated and maintained using appropriate culture conditions for each cell line. After 7 days of culture yellow tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma) assay was used for evaluation of NP cytotoxicity, following manufacturer instructions. Data is represented as the percentage mean relative to control optical density/control group \pm s.e.m. Each experiment was done in six replicates and repeated two times per cell line.

In vitro lymphocyte proliferation assay

Peripheral blood mononuclear cells (PMBCs) were provided from cell bank of the Centre for Scientific Instrumentation (CIC) of the University of Granada. Cryovials of PMBC were thawed according to manufacture instructions. Cells were resuspended in free serum DMEM at 1×10^6 cell/mL, and incubated 1 hour at 37°C horizontally. Monocytes were adherent to plastic flask and lymphocytes were collected from culture. After collecting lymphocytes from PBMC isolated cells, concentration was adjusted to 1×10^6 cells/mL and labelled with 25000 NP added per cell for 30 minutes at 37°C. Following washing steps, Cy5-NPs-labeled cells were resuspended in culture medium and divided into proliferating and resting cells. PHA-P was added to culture media in a final concentration of 2%. Cell proliferation was examined on days 0, 3, 5, and 7 after nanofection. Cy5-NP-labeled, unstimulated cells as a control, as well as stimulated cells

(both unlabeled and Cy5-NP-labeled) were analysed by flow cytometry. CFSE assay was performed according to the manufacturer's instructions as a control of monitoring proliferation lymphocyte model assay. Experiment was performed in quadruplicate from four independent PMBC batch.

Flow cytometry analysis

Dot plots and cytometry statistics were obtained using FlowJo software (Percentage of positive population and median of fluorescence intensity -MFI-) and FACS DiVa Software (BD Biosciences). Graphs and statistical difference data were performed using GraphPad Software according to the following explanation. Percentage data of cells containing Cy5-NPs were represented versus cell/Cy5-NPs ratio in a bar representation, and statistical significant differences were stabilised by two-way ANOVA Bonferroni's multiple comparison test in the different treatments between the same cell lines. Furthermore, median fluorescence intensity (MFI) was exhaustive analyzed comparing the MFI increment (Δ MFI, MFI sample/MFI untreated). Two-way ANOVA Bonferroni's multiple comparison test was applied to study statistical significance. A p-value of ≤ 0.05 was considered significant. Results are given as means \pm standard error of the mean (SEM). Sample size, which indicates experimental replicates from a single representative experiment, was 3 unless otherwise specified. The results of all experiments were validated by independent repetitions.