Chapter 11

Assessment of Cellular Uptake and Cytotoxicity of Carbon Nanotubes Using Flow Cytometry

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Abstract

The field of carbon nanotube (CNT) functionalisation is increasingly growing for the purpose of enhancing the biocompatibility of CNT for medical and biological applications. Properties of CNT such as the type of functionalisation, charge density, and the dispersibility profile are expected to modulate CNT cellular uptake and toxicity profile in vitro. The assay described here allows for rapid screening of CNT cellular uptake in vitro and assessing the acute cytotoxicity simultaneously. CNT cellular uptake is measured qualitatively by light scattering analysis without differentiating between cell binding and internalisation of the CNT by the cells. In addition, flow cytometry is used to combine light scattering analysis with flow cytometry-based Annexin V/propidium iodide assay to measure the cytotoxicity. This assay is rapid, reliable, and allows for comparative analysis between various types of CNT studied.

Key words: Carbon nanotubes, CNT, Association, Binding, Internalisation, Toxicity, Flow cytometry, Light scatter, Annexin V/PI, Apoptosis, Necrosis

1. Introduction

The emergence of carbon nanotubes (CNT) as advanced nanomaterials, and in particular towards biomedical and biotechnological applications is of great interest (1–4). Due to advancements in the available functionalisation chemistries of CNT and the development of new constructs of polymer-CNT assembly, a range of surface functionalised carbon nanotubes (f-CNT) of various types, charge densities, and dispersibility profiles have been generated (3, 5–10).

In terms of the biologically relevant features of CNT, one of the most attractive properties described is the capacity to translocate cellular barriers (such as the plasma membrane) by

K. Balasubramanian and M. Burghard (eds.), *Carbon Nanotubes: Methods and Protocols*, Methods in Molecular Biology, vol. 625,
DOI 10.1007/978-1-60761-579-8_11,

mechanisms that are novel and seem to be reminiscent of a nano-needle piercing the cells (11, 12). Numerous laboratories using various types of CNT have now reported their cellular uptake by a wide range of cells (11, 13–16). Therefore, a facile and rapid screening method to study the interaction of non-fluorescently labelled CNT with cells is needed. Some of the most popular techniques employed to study the interaction of CNT with mammalian cells in vitro include confocal laser scanning microscopy (17, 18), light microscopy, and transmission electron microscopy (19). Confocal laser scanning microscopy requires the tagging of CNT with fluorescent probes while transmission electron microscopy is a laborious process that not many laboratories may have access to and is not intended for routine use. Moreover, almost all microscopy-based techniques will always provide qualitative information with regards to the interaction between nanomaterials, including CNT, and cellular surfaces and compartments, with statistical analysis almost impossible to infer.

Flow cytometry-based assays have been proposed to assess cell-nanoparticle associations (both cell bound or internalised) either by quantitatively measuring the absolute number of fluorescent particles associated with cells or by qualitatively measuring the increase in the sideward scattering of cells incubated with non-fluorescent particles. Qualitative measurements are based on the fact that as nanoparticles bind to the cells, the granularity of the cells increases, which concomitantly increases the sideward scattering intensity.

Although internalisation of a variety of CNT into various types of cells can be seen as an attractive feature of these nanomaterials, particularly in relation to the delivery or detection of molecules intracellularly, there may be cytotoxic side-effects associated with such property that should be considered. In all cases that interaction between CNT and the biological milieu takes place, the ensuing cytotoxicity needs to be addressed. The new subfield of nanotoxicology has emerged in the last few years to specifically address and obtain a better understanding of the impact of novel nanoparticles and their health hazards.

The cytotoxicity of CNT has been assessed in vitro using methods that are well described in the literature (20, 21). They include direct counting of cell numbers through Trypan Blue exclusion assay (22), colorimetric assays such as the MTT (23, 24) or the LDH assay (23), measurement of protein concentrations by the Bradford assay (25), or by clonogenic assay (26). FACS and confocal microscopy cytotoxicity assays, such as the mitochondrial membrane potential determination and Annexin V/propidium iodide (PI) staining, have also been reported as methods to assess the cytotoxicity of CNT (22, 23, 27).

There have been conflicting conclusions from the assessment of the various types of CNT by different groups. One reason for

the discrepancies resulting from cytotoxicity assays is due to the variation in the impurities contained in different CNT preparations (i.e. metals such as iron and nickel in addition to the amorphous carbon content). Moreover, some studies have shown contradicting results when assessing the cytotoxicity of CNT by colorimetry-based assays such as MTT, WST-1, XTT or LDH assays (23). It has been already reported that the presence of CNT may lead to false positive results with the MTT assay due to the interaction between the water-insoluble MTT formazan crystals and the CNT backbone after MTT reduction by mitochondrial dehydrogenase of physiologically active cells. On the contrary, no interaction seems to occur between the CNT and the water-soluble formazan products produced by reduction of tetrazolium salts such as WST-1, XTT or INT which are the main components of the WST-1, XTT or LDH assays (23). Besides interactions reported in colorimetry-based assay, one has to be careful in fluorescence-based cytotoxicity assays, as interaction between CNT and fluorescent probes may occur based on the properties on the CNT studied (surface, length, and dispersibility), the fluorescent molecules used and the concentration range of the CNT tested. Another common issue arises from the fact that the total surface area available from the CNT is enough to adsorb reagent or fluorescent molecules (28), particularly those with many aromatic rings, therefore leading to false negative cytotoxicity results. There have been reports that CNT may also be able to quench the fluorescence of quantum dots (QDs), that are strongly and intrinsically fluorescent nanoparticles, through the formation of QD supramolecular assemblies around the CNT (29).

The assay presented in this method is designed to measure qualitatively by light scattering analysis, the CNT association with cells without differentiating between those nanotubes that bind on the cells to those that are internalised by the cells. In addition, flow cytometry is used to combine light scattering analysis with flow cytometry-based Annexin V/PI cytotoxicity assay to allow for simultaneous and rapid screening for CNT-cell association and cytotoxicity assessment.

2. Materials

1. Nanoparticle cell uptake experiments can be performed using adherent cells such as A549 lung epithelial cell line (CCL-185, ATCC, UK) or other cell lines. Cells should be removed from the culture dish before analysis with flow cytometry by trypsinisation.
2. Trypsin-EDTA (Gibco, Invitrogen, UK).

3. Culture media appropriate for the cell line studied. When A549 monolayers were used, the media used contained Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, UK), supplemented with 10% foetal bovine serum (FBS) (Gibco, Invitrogen, UK), 50 U/ml penicillin, and 50 µg/ml streptomycin (Gibco, Invitrogen, UK).
4. 24 Well Clear TC-treated microplates (Costar®, USA).
5. 1.5-ml microcentrifuge tubes.
6. 1, 5, and 25 ml serological pipettes (VWR, UK).
7. 10 µl, 200 µl, and 1 ml pipette tips (Starlab Ltd, UK).
8. 37°C and CO_2 incubator for maintaining the cells.
9. Centrifuge (350×*g* for 5 min) for pelleting cells.
10. Bath sonicator (Ultrasonic cleaner, VWR).
11. Annexin-V-Fluos staining kit (Roche Diagnostics GmbH, Germany), if toxicity experiments are needed. The kit contains ready-to-use Annexin V-FITC solution, PI solution, and HEPES incubation buffer.
12. CNT preparation to be tested, CNT functionalisation and dispersibility properties may vary. Pristine CNT can be used as an example of non-functionalised CNT.
13. Pluronic F127 co-polymer (Sigma) as dispersing agent for pristine CNT.

3. Methods

3.1. Preparation of CNT Dispersions

1. Disperse CNT powder in 5% dextrose, PBS or water, up to a maximum concentration of 1 mg/ml, by bath sonication for 15 min.
2. Store CNT dispersion in the fridge when not in use, and sonicate each time immediately before use.
3. Pristine CNT can be dispersed in 1% Pluronic F127 in water, aided by bath sonication for 15 min. Final concentration of Pluronic F127 when incubated with the cells should not exceed 1%.

3.2. Cell Culture

The following protocol describes the incubation of CNT with A549 lung epithelial cell lines. However, the type of cells can be changed based on the experimental design. The cells can be treated with various inhibitors, while the CNT can be modified by surface functionalisation or by changing the dispersing agent. The time of incubation with cells and CNT concentration can also be varied.

1. A549 cells should be passaged when they reach 80% confluence in order to maintain exponential growth and used for a maximum of 10 passages.
2. To trypsinise the monolayer, rinse it with PBS then incubate with trypsin-EDTA at 37°C for 5 min; the cells are then detached by vigorous up and down pipetting.
3. Centrifuge cells at 350×*g* for 5 min at 4°C and resuspend in complete media.
4. Count cells and determine cell viability by Trypan blue dye exclusion assay.
5. Adjust cell suspension to 100,000 cells/ml in complete media.
6. Seed 50,000 cells per each well of 24-well plates and incubate for 24 h at 37°C in a humidified atmosphere (5% CO_2) incubator.
7. Add CNT to 500 µl complete media in 1.5-ml microcentrifuge tubes, and vortex the suspension to mix. Use CNT concentrations between 1 and 100 µg/ml.
8. Allow the cells to interact with the CNT for 15 min, 60 min, 4 h, 24 h and 72 h, at 37°C in a humidified atmosphere (5% CO_2) incubator.
9. After incubation period is finished, aspirate media containing the CNT.
10. Add 500 µl of PBS to rinse the cells and remove unbound CNT (see Note 1).
11. Remove the adherent cells by adding 100 µl Trypsin-EDTA per well and incubate the cells at 37°C in a humidified atmosphere (5% CO_2) incubator.
12. Add 500 µl of tissue culture media, and detach the cells by vigorous up and down pipetting.
13. Transfer cells to 1.5-ml microcentrifuge tubes.
14. Centrifuge cells at 350×*g* for 5 min at 4°C and resuspend in PBS.
15. Keep cell suspensions on ice, and analyse immediately by flow cytometry.

3.3. Instrumentation and Gating

The scatter plots and gating have to be performed first. In the following methods described, the commands used are specific for the Summit version 4.3 for use with the CyAn™ ADP High-Performance Research Flow Cytometer (DakoCytomation, USA).

3.3.1. Setting a Bivariate Scatter Histogram

1. Set a bivariate sideward scattering (SS Lin) vs forward scattering histogram (FS Lin). The SS Lin should be displayed on the ordinate and FS Lin displayed on the abscissa (Fig. 1a).

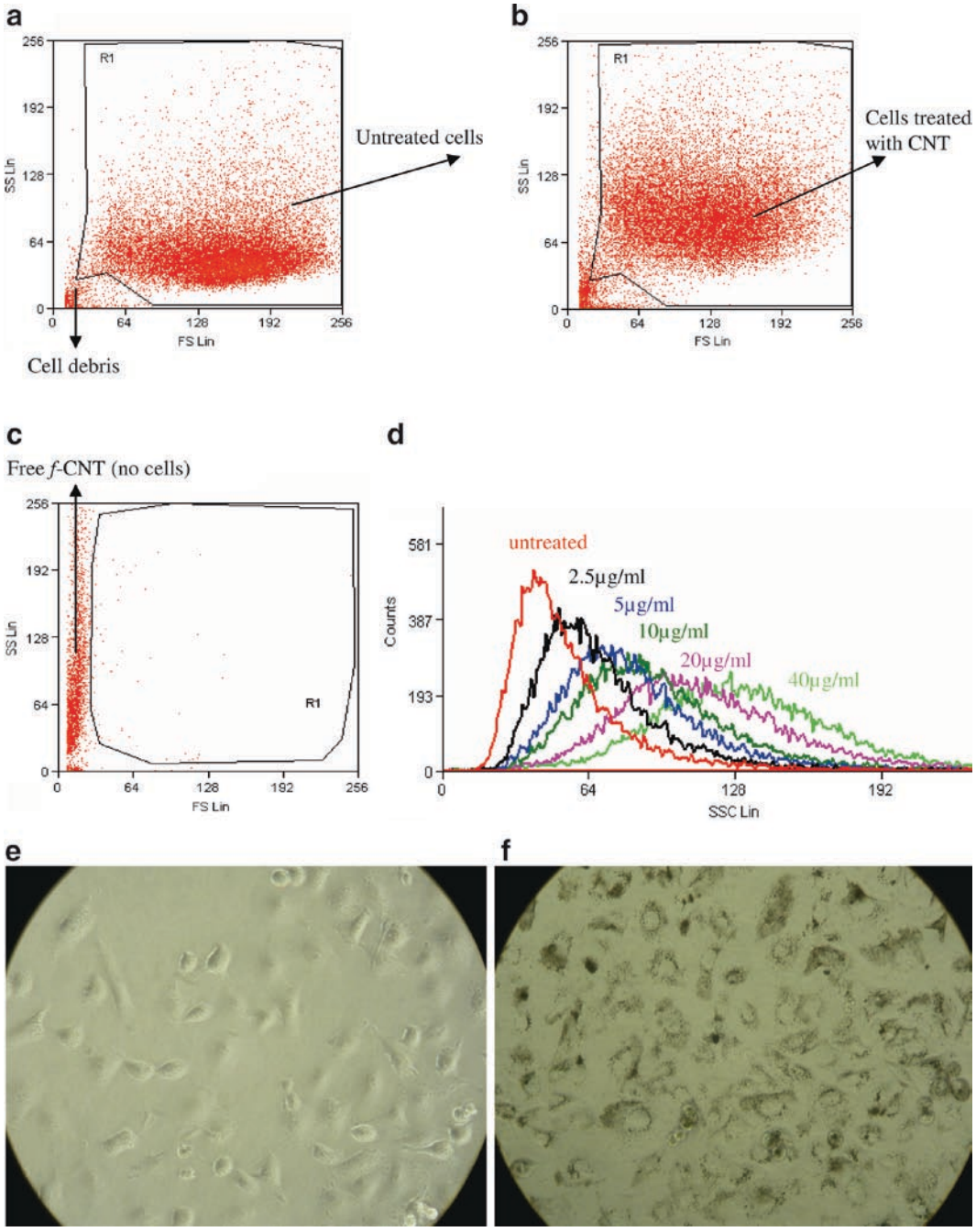

Fig. 1. A bivariate scatter histogram of the sideward scattering vs. forward scattering signals recorded with FACS for (**a**) cells incubated with media without CNT, (**b**) cells incubated with 10-µg/ml cationic f-CNT for 24 h, and (**c**) 10-µg/ml cationic f-CNT without cells. Each recorded event is presented as a point in the diagram. (**d**) A univariate scatter histogram showing the cell number (counts) vs. sideward scattering (SS Lin) for cells incubated with varying concentrations of the f-CNT for 24 h. (**e**) and (**f**) are light micrographs of untreated A549 cells and cells treated with 10-µg/ml cationic f-CNT for 24 h, respectively

2. The photomultiplier tube (PMT) voltage has to be set in a way that both the negative control (a sample containing cells without CNT) (Fig. 1a) and the positive control (a sample containing cells incubated with cationic CNT for 24 h) (Fig. 1b) have channel number from 0 to 265 for both ordinate and abscissa. If one cell type is used, only one cluster of cells will be observed (R1) and selected for setting a univariate scatter histogram (see Subheading 3.3.3).
3. In order not to include the CNT in the cell population group, a suspension containing the highest concentration of CNT without any cells should be run first to exclude any free CNT from R1 group (Fig. 1c).

3.3.2. Gating Cells from Bivariate Scatter Plot

1. Gating means an electronic gate (R1) should be selected inside the bivariate plot (Fig. 1). In this protocol, the gate R1 is selected to include all the cells being studied and exclude any cell debris (Fig. 1b) or free CNT (Fig. 1c).
2. Gate the cells to include cells over the entire range of the SS Lin channel (0–265). Cells interacting with CNT will have high SS Lin channel number, therefore, histograms of a positive and a negative control cells should be used when selecting the gate in order not to underestimate the SS Lin after incubation with CNT.
3. Adjust the optimum FS Lin (width) of the gate to remove the free unbound CNT and cell debris. Cell debris samples appear as a population with small FS Lin and small SS Lin (Fig. 1a). CNT appear as a population with small FS Lin and medium SS Lin (Fig. 1c). This step is important to minimise any interference from the CNT particles on both light scatter and toxicity data.

3.3.3. Setting a Univariate Scatter Histogram

Set a univariate histogram that plots cell number (counts) on the ordinate and SS Lin on the abscissa (Fig. 1d). The abscissa channel number should be set from 0 to 265. SS Lin should be gated from the cell population R1 in the bivariate plot (see Subheading 3.3.2).

3.3.4. Measuring SS Lin of the Samples

1. Measure the SS Lin of a sample containing the CNT alone at the highest concentration used in the experiment. Verify that gate R1 set in the bivariate sideward scattering (SS Lin) vs. forward scattering histogram (FS Lin) exclude all the unbound CNT.
2. Measure the SS Lin of the negative control samples (cells without CNT) and set the SS Lin channel number to around 50 (0–256 scale).
3. Measure the SS Lin of the positive control samples (cells incubated with cationic CNT for 24 h). Verify that gate R1

set in the bivariate sideward scattering (SS Lin) vs. forward scattering histogram (FS Lin) include all the cells and exclude the free CNT.

4. Analyse at least 20,000 cells per sample. Record the median SS Lin from the univariate sideward scattering (SS Lin) set in Subheading 3.3.3.
5. Cell-association data will be analysed as in Subheading 3.3.5 (see Notes 2 and 3).

3.3.5. Data Analysis

Typical result from the 2D density plot of the forward scattering and sideward scattering is shown in Fig. 1a. High forward scattering events correspond to large particles such as cells (Fig. 1a and b) whereas smaller forward scattering events correspond to smaller particles such as CNT (Fig. 1c) or cell debris (Fig. 1b). Taking into account the events with high forward scattering events (cells), high sideward scattering events correspond to cells that are associated with CNT which can either be bound to the cell membrane or internalised by the cell (Fig. 1b). On the other hand, low sideward scattering events correspond to cells without associated CNT (Fig. 1a). Data analysis is done by comparing the sideward scattering intensity of control cells (no CNT) to that of cells incubated with CNT. The median sideward scattering intensity from the univariate sideward scattering histogram can be used for comparison (Fig. 1d). Data are generally expressed as fold changes in median sideward scattering intensity for a given CNT/cell sample as compared to that of control cells.

3.4. CNT Cytotoxicity by Flow Cytometry

Cell death is known to occur by two distinct modes: necrosis and apoptosis (30, 31). Although morphological, biological, and molecular differences between necrosis and apoptosis are evident, the boundaries between necrosis and apoptosis are not always very clear as the patterns of biochemical or morphological changes are not always of typical necrosis or apoptosis. The light scattering properties of cells during death can change due to morphological changes such as cell swelling, cell shrinkage, rupture of the plasma membrane, chromatin condensation, nucleus fragmentation, and shedding of apoptotic bodies. Necrotic death is characterised by rapid initial increase in forward and sideward scattering due to cell swelling. Apoptotic death is characterised by a decrease in both forward and sideward scattering, however, an initial increase in sideward scattering parallel with a decrease in forward scattering has been observed in some cell types (32, 33). In general, broken cells, isolated nuclei, cell debris, and apoptotic bodies have low light scatter properties. Since light scatter analysis is specific to neither apoptosis nor necrosis, more mechanistic data can be obtained by combining light scatter analysis to another cytofluorimetric analysis such as Annexin V/PI staining.

3.4.1. Annexin V/PI Staining

In this assay, we combine analysis of CNT-cell association by light scatter changes with another cytofluorometric cytotoxicity assay for the detection of phosphatidylserine with Annexin V-FITC conjugate. In early stages of apoptosis, the plasma membrane phospholipid, phosphatidylserine (PS), is exposed to the outside of the plasma membrane (34). Annexin V is a Ca^{++}-dependent phospholipid-binding protein, which binds to PS residues. Annexin-V-FITC conjugate can be used to detect apoptosis. Since PS externalisation may also happen during cell necrosis, including membrane impermeable dye such as PI can distinguish apoptotic cells from necrotic cells. Non-apoptotic non-necrotic cells are Annexin V-negative ($FITC^-$) and PI-negative (PI^-), early apoptotic cells are Annexin V-positive and ($FITC^+$) and PI-negative (PI^-), and late apoptotic cells and necrotic cells are intensely stained with PI.

If cytotoxicity assessment is required, follow steps 1–13 (see Subheading 3.2) and continue as below:

1. Aspirate PBS from cell pellets.
2. Add 500 µl of PBS to remove any serum traces.
3. Centrifuge cells at 350×*g* for 5 min at 4°C.
4. Prepare Annexin V-FITC/PI labelling solution as instructed by the manufacturer. Mix 98 µl of HEPES incubation buffer with 1 µl of ready-to-use Annexin V-FITC and 1 µl of ready-to-use PI solution.
5. Keep three tubes with cells that are unlabelled, labelled with Annexin V-FITC only or PI only, to set the photomultiplier tube (PMT) voltage and compensation settings.
6. Aspirate the PBS from each 1.5 ml microcentrifuge tubes and add 100 µl of the above solution to each tube.
7. Resuspend the cell pellet in 100 µl of Annexin V-FITC/PI labelling solution, and incubate for 10–15 min at 15–25°C, then keep cell suspensions on ice and analyse immediately by flow cytometry.
8. Just before analysis, dilute the cell suspension with 0.4 ml of HEPES incubation buffer and transfer to a test tube for analysis directly on the flow cytometer.

3.4.2. Flow Cytometry

1. Samples are analysed on a flow cytometer using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter of 615 nm for PI detection.
2. Electronic compensation of the instrument is performed to exclude overlapping of the two emission spectra.
3. At least 20,000 cells per sample are analysed.
4. Cytotoxicity data will be analysed as in Subheading 3.4.3.

3.4.3. Data Analysis Cell death was expressed as percentage cell population stained with Annexin V-FITC or with PI staining (see Note 4).

4. Notes

1. CNT may bundle and if the size of the bundle is not small enough (i.e. less than 2 μm), they will be counted as unstained cells leading to false negative results. Therefore, it is highly recommended that the cytotoxicity of CNT needs has to be verified using at least two independent cytotoxicity assays.
2. This is a qualitative rather than a quantitative assay that measures CNT-cell association, in contrast to assays using fluorescent CNT where precise quantification of the percentage of internalised CNT may be feasible.
3. From Fig. 1b, it is difficult to distinguish between the CNT that are bound to the cell surface or internalised by the cells because sideward scattering intensity is an indication of surface roughness of the cell. However, this technique can be combined with other techniques such as light microscopy, transmission electron microscopy, or confocal laser scanning microscopy to distinguish CNT cell binding from internalisation. Light scattering analysis is being proposed here as a screening tool to study the effect of varying CNT characteristics on their interaction properties with cells. CNT of different surface charge, charge density, and dispersibility profile can be tested comparatively. Data generated using light microscopy established a good correlation between the increase in sideward scattering intensity and the increase in CNT intracellular accumulation (Fig. 1e and f), which suggested that adsorption of the CNT onto the cell membrane will eventually lead to intracellular uptake.
4. A 2D density plot of the green (apoptosis) and red fluorescence (necrosis) is shown in Fig. 2. The events shown in Fig. 2 are gated to the cell events (R1) chosen in Fig. 1. The diagram can be divided into four populations. Events with low red and low green fluorescence (bottom left quadrant) correspond to non-apoptotic non-necrotic cells, events with low red and high green fluorescence (bottom right quadrant) correspond to early apoptotic cells, events with high red and high green fluorescence (top right quadrant) correspond to late apoptotic or necrotic cells, and events with high red and low green fluorescence (top left quadrant) correspond to nuclear fragments. Integration over all events in each quadrant yields the total number of events.

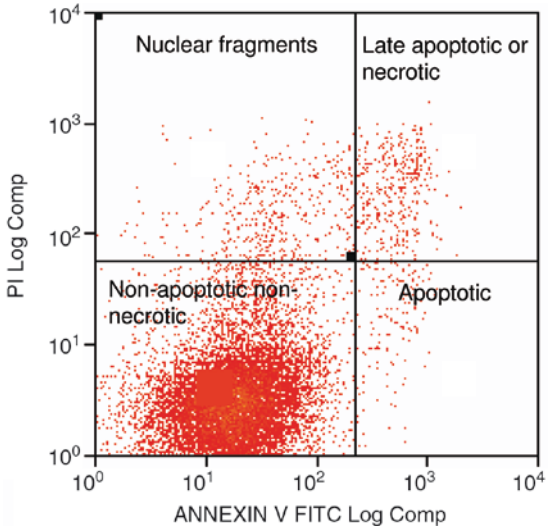

Fig. 2. Evaluation of cell viability using Annexin V/PI staining. All events are gated to R1 population obtained in Fig. 1. The diagram can be divided into four populations. Events with low red and low green fluorescence (*bottom left quadrant*) correspond to non-apoptotic non-necrotic cells, events with low red and high green fluorescence (*bottom right quadrant*) correspond to early apoptotic cells, events with high red and high green fluorescence (*top right quadrant*) correspond to late apoptotic or necrotic cells, and events with high red and low green fluorescence (*top left quadrant*) correspond to nuclear fragments

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