Chapter 20

High-Accuracy Determination of Cytotoxic Responses from Graphene Oxide Exposure Using Imaging Flow Cytometry

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Abstract

Graphene and other 2D materials have received increased attention in the biomedical field due to their unique properties and potential use as carriers for targeted drug delivery or in regenerative medicine. Before the exploitation of graphene-based materials in biomedicine becomes a reality, it is necessary to establish the full toxicological profile and better understand how the material interacts with cells and tissues. Because specific properties, such as flake size and surface chemistry, might determine whether graphene can achieve therapeutic efficacy without causing toxicity, it is important to develop highly accurate and reliable screening techniques to accurately assess the biocompatibility of different types of graphene-based materials. In this protocol, we describe a method to achieve accurate determination of the cytotoxic response following in vitro exposure to large graphene oxide (L-GO) sheets using annexin V/propidium iodide staining and the Imagestream® platform. The proposed protocol is especially suitable for the toxicity assessment of carbonaceous materials that form aggregates in cell culture media, which is a common occurrence. We describe how to best gate out any interfering signals coming from the material by visual inspection and by using powerful software, thus performing the analysis of cellular death on a selected population of cells with higher accuracy and statistical relevance compared to conventional flow cytometry.

Key words Graphene oxide, Imagestream, Imaging flow cytometry, Toxicity, Annexin V/propidium iodide, Cell viability assay, 2D material, Material agglomeration, Apoptosis, Necrosis

1 Introduction

Graphene-based nanomaterials started receiving considerable attention due to their unique chemical and physical properties, such as enhanced electron mobility and thermal conductivity, mechanical strength, and distinctive optical characteristics, which can be exploited in biomedicine [1]. Graphene and other 2D materials can be used as carriers for targeted drug delivery, cancer treatment agents *via* photo-thermal therapy, or scaffolds for nerve regeneration [2, 3]. However, in vitro and in vivo knowledge regarding the safety and biocompatibility of graphene-based materials is still being gathered. Such studies are critically important to provide better insight into the interactions of 2D materials with

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cells and tissues, thus helping us to further understand their biological safety profile.

The assays used to determine cytotoxicity of graphene-based nanomaterials in vitro are similar to those developed for other carbon-based nanostructures (e.g., carbon nanotubes) and for other types of nanomaterials in general. The most commonly used cytotoxicity tests are colorimetric assays, such as the "modified" lactate dehydrogenase (LDH) and water-soluble tetrazolium salts (WST-8) assays; these are considered to be the most reliable because potential interferences of nanomaterials with the components of the assay might be avoided [4–6]. Carbon-based materials have repeatedly been found to interfere with the MTT assay, which is also widely used to determine cytotoxicity induced by nanomaterial exposure [6, 7]. More sophisticated techniques, such as flow cytometry, can offer higher accuracy, but often with a lower throughput [8]. The advantage of flow cytometry is that the sampled cells can be sorted based on different parameters such as their size, granularity, or fluorescence, all of which might be influenced by the interactions between cells and the nanomaterial. A popular flow cytometry-based assay used to study the cytotoxicity of carbonaceous nanomaterials involves annexin V/propidium iodide staining, which we described previously [9]. Using this technique, unstained events on flow cytometry bivariate plots are distinguished from the stained events. The unstained events are considered to be live cells, annexin V-positive events belong to early apoptotic cells, propidium iodide-positive events indicate necrotic cells, while both annexin V and propidium iodide-positive events represent either late apoptotic or necrotic cells.

A potential issue in assessing the cytotoxic responses of cells to carbon-based materials in vitro using flow cytometry comes from the fact that the material commonly tends to agglomerate when dispersed in cell culture media or after interacting with molecules secreted by the cells [10, 11]. The size of such agglomerates can be similar to the size of a cell and therefore can appear as an "unstained event" on the annexin V/propidium iodide bivariate plot. Subsequently, this results in an overestimate of the number of live cells in the sample, which can lead to inaccurate conclusions regarding the cytotoxicity of the material [9]. Gating systems provided in classic flow cytometry software offer the possibility of excluding such interferences; however, the accuracy of the gating is limited as the events being gated in or out cannot be visually inspected.

More advanced flow cytometry techniques and instrumentation, such as Imagestream®, are combining flow cytometry with high-resolution imaging, therefore providing both statistical power over the acquired data in conjunction with the possibility of viewing each individual acquired event. Using this technique, it is possible to distinguish whether each acquired event in the bivariate plot is the result of an aggregated material or a cell. Moreover, using

further software analysis, it is possible to gate out the material based on its contrast properties (the contrast properties of aggregated materials and cells differ). The combination of those capabilities offers superior accuracy in comparison to classic flow cytometry and colorimetric assays. Imagestream® has so far been used to accurately assess the uptake of different nanomaterials, including carbon nanotubes in vitro [11–14] as well as to determine the extent of cell death induced by different pharmacological agents [15, 16].

In this protocol, we describe a method that can be used to accurately determine the cytotoxic responses of mammalian cells following exposure to large (>2 μm and <20 μm in lateral dimension), but thin (1–2 layers, 0.6 nm thickness of one layer) graphene oxide (GO) sheets using annexin V/propidium iodide staining and Imagestream®. We provide an explanation on how best to gate out any interfering signals from the material itself by visual inspection and by applying the software features provided with the instrument, and in this way, to perform analyses of cellular death on a selected population of cells with high accuracy and statistical relevance. The proposed protocol is suitable for the assessment of the toxicity of carbonaceous materials that form aggregates in the cell culture media; however, it can be further optimized and used for any type of carbon- or non-carbon-based material.

2 Materials

2.1 L-GO Preparation

1. Large GO (L-GO) material dispersed in sterile, endotoxin-free water (2.4 mg/mL).
2. RPMI 1640 cell culture medium with L-glutamine and sodium bicarbonate (R8758, Sigma-Aldrich, Merck, UK).
3. Fetal Bovine Serum (FBS, Sigma-Aldrich, Merck, UK).
4. 15 mL sterile, plastic tubes (Corning, Costar, Sigma-Aldrich, Merck, UK).
5. Vortex.

2.2 Cell Culture

1. Adherent immortalized lung epithelial cell line Beas-2B (CRL-9609, ATCC).
2. Cell culture medium appropriate for the cell line studied. For the Beas-2B cell line, the RPMI 1640 cell culture medium with 20 mM glutamine (R8758, Sigma-Aldrich, Merck, UK) and supplemented with 10% FBS (Gibco, Thermo Scientific, UK), 50 U/mL penicillin, and 50 μg/mL streptomycin (all from Sigma-Aldrich, Merck, UK) was used.
3. 0.05% trypsin with 0.53 mM ethylenediaminetetraacetic acid (EDTA) tetra-sodium salt (T3924, Sigma-Aldrich, Merck, UK).

4. Six-well flat-bottom plates (Corning, Costar, Sigma-Aldrich, Merck, UK).
5. T-75 sterile flasks (Corning, Costar, Sigma-Aldrich, Merck, UK).
6. Incubator set at 37 °C and 5% CO_2.
7. 1.5 mL micro centrifuge tubes.
8. 5, 10, and 25 mL serological pipettes (VWR, UK).
9. 10 μL, 200 μL, and 1 mL pipette tips (Starlab, UK).
10. Centrifuge (210 × *g* for 5 min) for pelleting cells.
11. 15 mL sterile, plastic tubes (Corning, Costar, Sigma-Aldrich, Merck, UK).
12. Annexin V, Alexa Fluor® 488 conjugate (A13201, Thermo Fisher Scientific, UK).
13. Annexin Binding Buffer (V13246, Thermo Fisher Scientific, UK).
14. Propidium iodide (P4864, Sigma Aldrich, UK).
15. 5% dimethyl sulfoxide (DMSO) (D2650, >99.7%, sterile, filtered, Sigma-Aldrich, Merck, UK).
16. Dulbecco's Phosphate Buffered Saline (PBS), with $MgCl_2$ and $CaCl_2$ (D8662, Sigma-Aldrich, Merck, UK).
17. Trypan blue (T8154, 0.4% solution, Sigma-Aldrich, Merck, UK).

3 Methods

This protocol allows determination of cytotoxic responses from the exposure to L-GO material incubated with Beas-2B cells for 24 h. The protocol is especially suitable for materials that contain one structural dimension at the micron scale or smaller-sized nanomaterials that form aggregates of sizes similar to that of a cell, which biases quantitative assessment of toxicity. Time points and concentrations of treatment as well as the cell type or the type of the material and its surface functionalization can be modified (*see* **Note 1**).

3.1 Preparation of L-GO Dispersions

1. Synthesize L-GO sheets from graphite powder (Sigma-Aldrich) according to a previously described modified Hummers method and purification protocols [8, 17]. The lateral dimensions of the L-GO flakes are between 2 and 20 μm, with a thickness ranging between 1 and 2 layers. Disperse L-GO material in complete cell culture medium (RPMI1640 cell culture medium + 10% FBS) to obtain a concentration of 0.05 mg/mL, which is the highest concentration of treatment

for the cells (*see* **Note 2**). Prepare the dispersion shortly before the treatment in a 15 mL sterile, plastic tube. Vortex thoroughly immediately after the preparation and again before treating the cells.

2. In order to determine the concentration of the L-GO that is inducing a significant decrease in cellular viability compared to the untreated cells, perform a dose escalation study. Prepare successive dilutions of the material (0.025 and 0.0125 mg/mL) by diluting a concentrated solution of the material (0.05 mg/mL) with complete cell culture medium in 15 mL sterile, plastic tubes.

3.2 Cell Culture Treatment and Preparation for Data Collection

1. Grow the cells in T-75 flasks in complete cell culture medium until they reach 80% confluence, and then passage them. In order to detach cells from the support, rinse them first with 1 mL of trypsin-EDTA at 37 °C. Incubate the cells with 3 mL of trypsin-EDTA at 37 °C for no longer than 5 min.
2. Detach cells by up and down pipetting, then place them in a 15 mL sterile tube, and add 10% FBS (300 μL) to stop the action of trypsin-EDTA.
3. Count cells and determine the number of live cells per mL using a trypan blue dye exclusion assay.
4. Seed 20,000 cells/cm^2 in six-well plates, using 2 mL of complete cell culture medium per well, and incubate them for 48 h to allow the cells to reach 80% confluence (*see* **Note 3**, *see* Fig. 1 for a schematic of the cell preparation and treatment protocol).

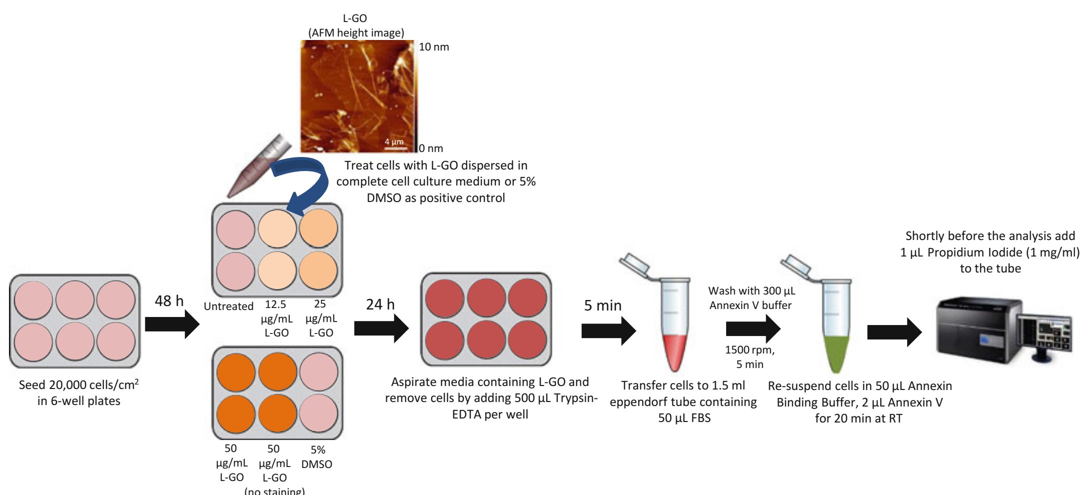


Fig. 1 Schematic of the cell treatment protocol and staining procedure. Atomic Force Microscopy (AFM) height image shows lateral dimensions and thickness of L-GO flakes used for the treatment

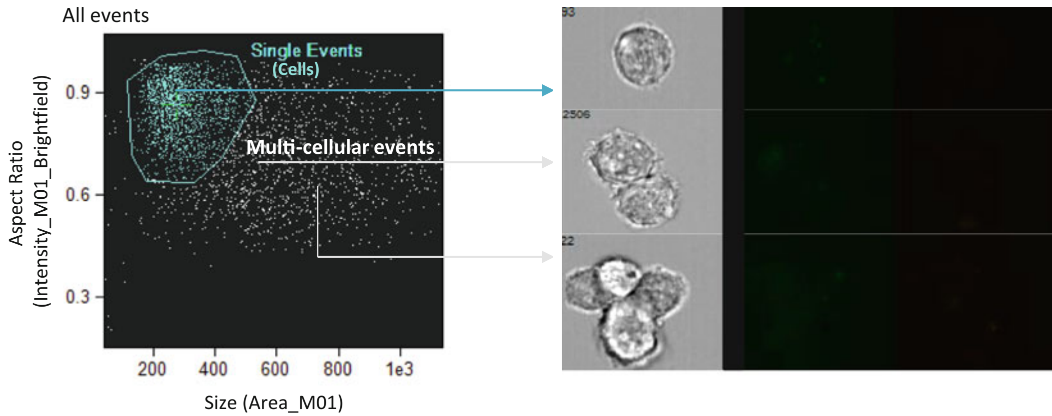
5. Prepare the following controls, which will ensure proper setup of the instrument laser and successful subsequent data analysis: (1) untreated (complete cell culture medium will be added) and unstained cells (the background autofluorescence control), (2) untreated (complete cell culture medium will be added) cells, stained with annexin V/propidium iodide (the negative control for the dose escalation study), (3) cells treated with the highest concentration of the material (0.05 mg/mL) (*see* Subheading 3.1, **step 2**), but left unstained (necessary to determine if, upon interaction with the cells, the material emits signal when excited with the same laser used to excite the annexin V/propidium iodide dyes), (4) cells treated with 5% DMSO and stained with annexin V and cells treated with 5% DMSO and stained with propidium iodide (the single-stained positive controls, necessary to create a compensation matrix and remove spectral overlap), and (5) cells treated with 5% DMSO and stained with both annexin V and propidium iodide (a double-stained positive control, necessary for the setup of the excitation laser of the instrument and as a positive control for the dose escalation study).
6. Prepare the samples. Treat the cells with 3 mL of the L-GO material dispersed in complete cell culture medium (*see* Subheading 3.1, **step 2**). These will be stained with both annexin V and propidium iodide to carry out a dose escalation study.
7. After treatment, incubate the cells at 37 °C in a 5% CO_2 humidified atmosphere for 24 h.
8. After the incubation period is finished, aspirate the media from all of the samples and controls (*see* **Note 4**).
9. Remove the adherent cells by adding 500 μL trypsin-EDTA to each well and incubate the cells at 37 °C for 5 min in a humidified atmosphere.
10. Detach the cells from the support by up and down pipetting and transfer the cells from one well to a 1.5 mL Eppendorf tube containing 10% FBS (50 μL) to stop the action of trypsin-EDTA (*see* **Note 5**).
11. Centrifuge cells at 210 × *g* for 5 min.
12. Carefully remove the supernatant and gently resuspend the cells in 300 μL of 1× Annexin Binding Buffer to wash them (*see* **Notes 6** and **7**).
13. Centrifuge the cells at 210 × *g* for 5 min.
14. Remove the supernatant and resuspend the cells in 50 μL of Annexin Binding Buffer (*see* **Note 8**).
15. For those samples and controls that require annexin V staining, add 2 μL of annexin V-Alexa 488 to each tube.

16. Incubate the cells in the dark at room temperature for 20 min.
17. Place cell suspensions on ice until analysis.
18. Shortly before the analysis, for those samples and controls that also require propidium iodide staining, add 1 μL of propidium iodide (1 mg/mL) to the tubes.
19. Acquire the data.

3.3 Data Collection (INSPIRE Software)

The Imagestream® platform processes the data in two steps: data are first acquired using the Amnis INSPIRE™ application provided with the Imagestream® instrument. Next, the IDEAS software, which can be freely downloaded, processes and analyzes the data. This software contains the algorithms and tools required to analyze the images acquired using the INSPIRE application in the first step. Compensation for the spectral crosstalk needs to be calculated from the control single-stained files and applied to all of the experimental files.

1. Turn lasers on according to the excitation/emission spectra of the dyes used. For this protocol, the lasers turned on are: 488 nm (for the excitation of annexin V and propidium iodide) and 785 nm (the side-scatter and bright-field laser).
2. Start the acquisition by running the brightest sample first. In this protocol, we first run the positive control for the cell death (i.e., cells treated with 5% DMSO and subsequently stained with both annexin V and propidium iodide). This step is critical to establish settings of the excitation laser power and to avoid saturation of the fluorescent signal. For the excitation of annexin V and propidium iodide, 488 nm laser power was set at 60 mW, while 785 nm laser was set at 0.02 mW. To ensure the accuracy of the results, the same laser power settings must be used for all of the samples.
3. Select 60× magnification and acquire images with a normal depth of field.
4. Turn on the appropriate fluorescence emission channels. Channel 01 is used for the bright field, Channel 02 for annexin V and Channel 04 for propidium iodide.
5. Create a bivariate plot to gate the cells. This plot should have the "Area_M01" feature on the *x*-axes and "Aspect Ratio Intensity_M01_Brightfield" feature on the *y*-axes. This enables the population of single events to be gated in the analysis and eliminates doublets or signals from debris (Fig. 2). Before starting the acquisition, make sure that at least 5000 events will be acquired.
6. After running the positive control, run the untreated cells (i.e., the untreated and stained cells as a negative control for the dose escalation study and the untreated and unstained cells for the



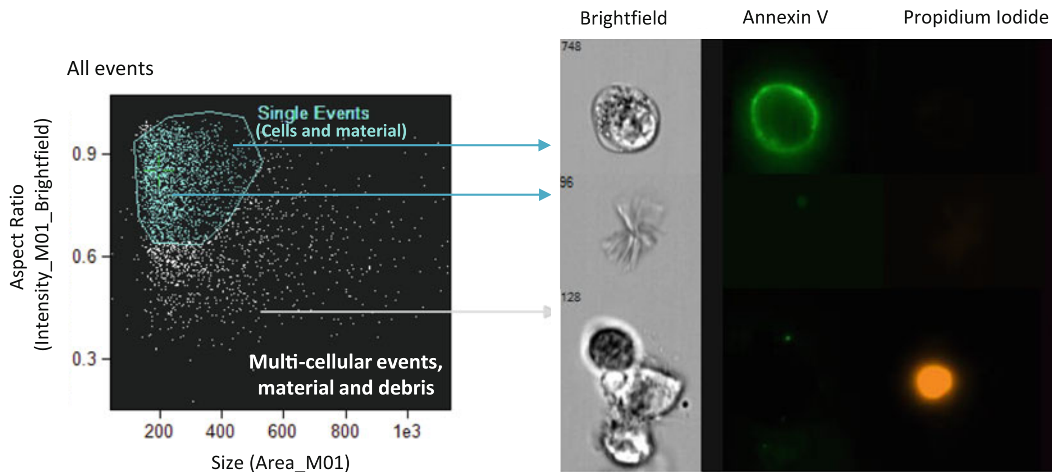


Fig. 2 Bivariate plots and gating applied to distinguish single events in the population of all acquired events of untreated (**a**) and cells treated with 50 μg/mL L-GO (**b**). The aim of this step is to exclude doublets, multicellular events, and debris from the analysis. Bivariate plot is distinguishing events based on their size using "Area_M01" and "Aspect Ratio Intensity_M01_Brightfield" features provided in the IDEAS software. This separation is based on a Brightfield image using a mask that encompassed whole cell (M01). "Area_M01" is the size of the event in a Brightfield image expressed in square microns and "Aspect ratio" is a measure of the circularity of the event. Aspect ratio of 1 corresponds to a perfect circle (such as rounded single cell), while doublets have Aspect ratio of 0.5. Note that in the population of cells treated with 50 μg/mL L-GO (**b**) gated single events will include not only single cells, but also the material. This can be seen in Imaging Gallery after clicking on a corresponding event on the bivariate plot

autofluorescence check), as well as the cells treated with the highest concentration of the material but left unstained. Finally, run the cells treated with escalating doses of the L-GO material (the samples).

7. The single-stained positive controls should be run last to record files for the compensation matrix (*see* Subheading 3.4, **step 1**). For this purpose, follow the instructions in the "Compensation" tab. Briefly, 500 events need to be acquired with all the channels turned on (except the bright-field and dark-field channels).

3.4 Data Analysis (IDEAS Software)

1. Start the data analysis by creating a compensation matrix by following the instructions in the "Compensation" tab. When clicking the "Create new matrix" tab, it will be required to insert the files acquired using the single stains only. The compensation matrix will be automatically generated by IDEAS software. Save it to apply it to all other acquired data files.
2. Create bivariate plots to gate the cells. The first plot should have the "Area_M01" feature on the *x*-axes and "Aspect Ratio Intensity_M01_Brightfield" feature on the *y*-axes. This enables the user to gate the population of single events in the analysis and eliminate doublets or signals from debris (Fig. 2).
3. Create a second bivariate plot based on the single events, selected in Subheading 3.4, **step 2**. This plot will gate the events that are in focus and distinguish them from the events that are not in focus, including the material under study. This step is crucial and a prerequisite for subsequent image-based analysis using IDEAS software. The *x*-axes are labeled "Gradient RMS_M01_Brightfield" and the *y*-axes are labeled "Contrast_M01_Brightfield." Selected events should have high values of gradient and contrast features and should be inspected in the Imaging Gallery before including or excluding them from the gate. All the events with high values of the contrast and gradient will be gated as "Focused events." In order to confirm that selected events include only cells in focus and not the material, inspect all events included in the gate in the preview option in Imaging Gallery (*see* **Note 9** and **10**). Readjust the gate if necessary (Fig. 3).
4. After events involving cells have been selected and separated from those involving nanomaterials, create third bivariate plots using "Focused cells" with the "Intensity_MC_Channel_02" (annexin V) on the *x*-axes and "Intensity_MC_Channel_04" (propidium iodide) on the *y*-axes. Draw the gates using the "Untreated cells" file and create four gates: AV-/PI- (alive cells), AV+ (early apoptotic cells), PI+ (necrotic cells), and AV+/PI+ (late apoptotic and/or necrotic cells). Click the symbol "Σ" in the upper right corner of the bivariate plot. The number of cells and percentages in each gate will appear (Fig. 4).

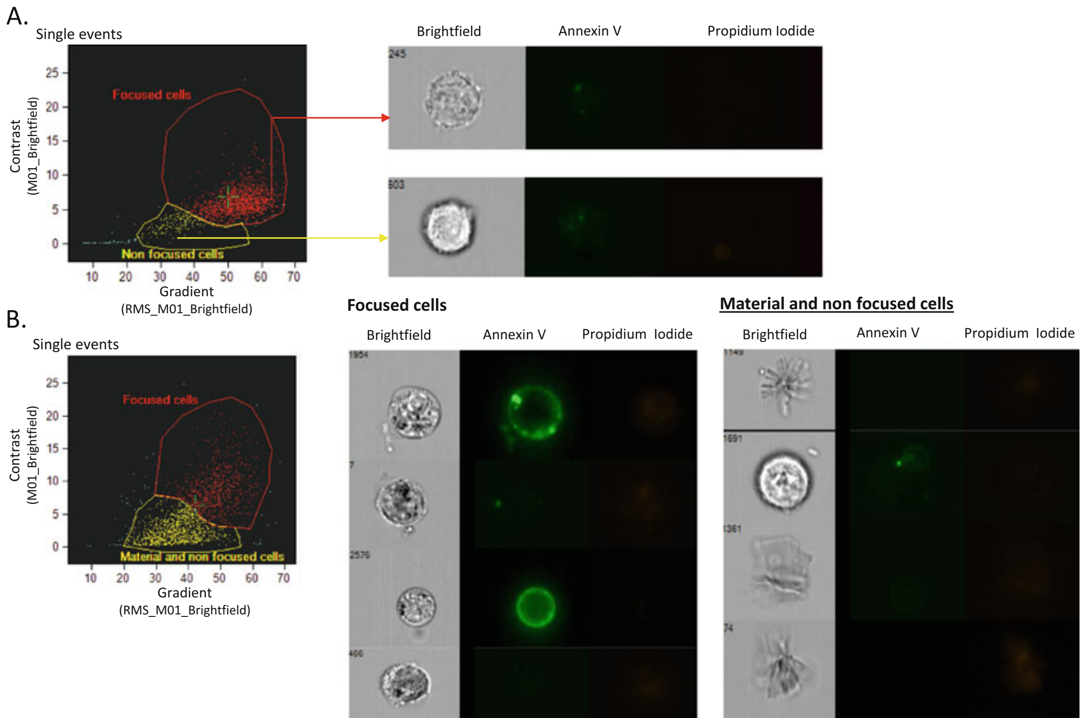


Fig. 3 Bivariate plot and gating used to select cells in focus and separate them from the material and nonfocused events in untreated (**a**) and cells treated with 50 μg/mL L-GO (**b**). Features for the bivariate dot plot are calculated based on a Brightfield image and using the mask that covers whole cell (M01_Brightfield extension in the name of a feature). As already described, "Contrast_M01_Brightfield" and "Gradient RMS_M01_Brightfield" values for a cell change after interaction with carbon-based materials [13]. Low Gradient RMS (root mean square) and Contrast feature values characterize events that are unfocused, which is the case for some cells and all the material in the analysis. Gating of the events with high values of these features enables to select cells that will be included in the analysis of the cellular death and separate them from the material. Successful separation of the cells from the material needs to be verified in Imaging Gallery by observing all gated cells

5. Once all three plots are created for one experimental condition, create a "Statistic report" template with parameters including percentage of double negative, annexin V single positive, propidium iodide single positive, and annexin V/propidium iodide double positive cells. This sheet can be saved as a template and then applied to all the other control samples using the "Batch Data Files" option in the "Tools" tab.
6. Before exporting the values of all files and plotting them in graphs, make sure that gates are set properly in each of the files. Once the gating is readjusted, export the values and create a graph (Fig. 5, *see* **Note 11**).

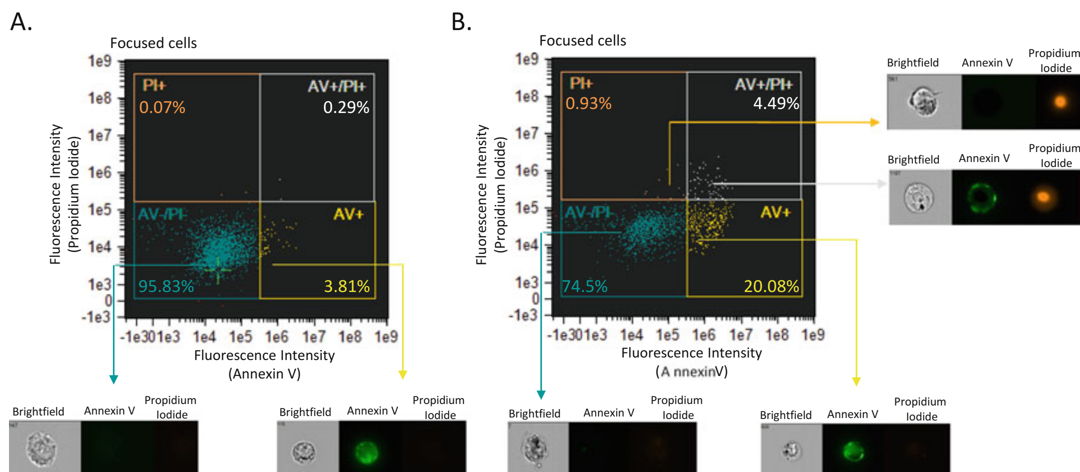


Fig. 4 Analysis of cellular death of untreated (**a**) and cells treated with 50 μg/mL L-GO (**b**) using annexin V/ propidium iodide staining based on a population of single, focused cells. Bivariate plot includes intensity of fluorescence collected using channel for annexin V (Intensity_MC_Annexin V) and propidium iodide (Intensity_MC_Propidium Iodide). Gates for double negative, single and double positive cells are designed based on a population of "untreated and stained" cells and then applied on all other treatment conditions. Images of cells corresponding to different gates can be previewed in Imaging Gallery (*insets*). Percentage of cells in each gate are calculated by the software and can be found after clicking on a "Σ" symbol in the upper right corner of a plot

4 Notes

1. This cell line is relevant to study cytotoxicity in in vitro models representing the exposure to the material by inhalation. Other adherent and nonadherent cell lines (such as A549, MCF-7, MH-S, THP-1, etc.) can be used as well. Cells should be removed from the plate before analysis using trypsin.
2. The highest dose of the L-GO material used for this experiment was 0.05 mg/mL. Higher doses tend to stick to the surface of the cells and quench the fluorescence of the dyes, thus indicating that the material could be less toxic than it really is.
3. It is important to grow and treat cells on six-well plates or larger surfaces to collect enough cells for the analysis. It is required to have at least 10^6 cells per sample. Treat the cells when they have reached 80% confluence if six-well plates are used; otherwise, the number of collected cells might not be sufficient.
4. After treatment with the material, GO in this case, it is important to remove the supernatant before collecting the cells to prevent the quenching of the fluorescence of the dyes due to the interference of the material with the fluorochrome.
5. The cells can be stained and fixed with paraformaldehyde if the analysis cannot be performed immediately following the

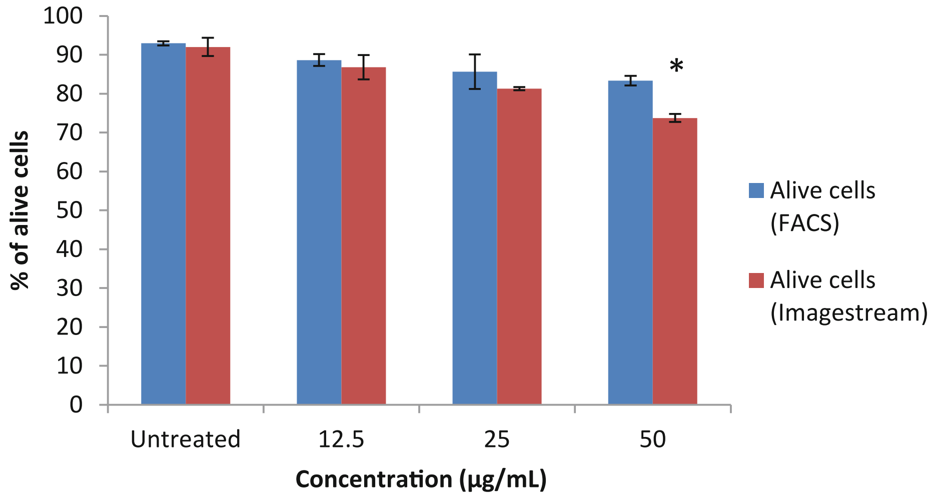


Fig. 5 Comparison of cellular viability assessed using FACS and Imagestream®. Cells were treated with increasing concentrations of L-GO material dispersed in the complete cell culture medium for 24 h, collected and stained using described annexin V/propidium iodide protocol. Cellular viability was assessed either using flow cytometry (FACS Verse instrument) or imaging flow cytometry (Imagestream®). Higher accuracy of the cytotoxicity assessment was achieved using imaging flow cytometry compared to flow cytometry, especially after treatment with the highest concentration of L-GO material due to a possibility of observing the events included in the analysis and of excluding aggregated material from the analysis. Data are represented as means ±SD ($n = 6$) and were statistically analyzed with IBM SPSS software (version 22) using analysis of variance (one-way ANOVA) with $p < 0.05$ considered significant

treatment. Store the fixed cells at 4 °C. However, because the washing and centrifugation steps required for fixation may introduce further cellular damage, the analysis of nonfixed samples is preferred.

6. If necessary, cells can be carefully washed using Phosphate Buffered Saline (PBS) with $MgCl_2$ and $CaCl_2$; however, during the washing and depending on the cell type, the cells might detach from the support, decreasing the population of analyzed cells. Using this protocol, cells can be analyzed without washing as the material removed from the surface is efficiently excluded from the analysis.
7. Carefully remove the supernatant after the centrifugation step in order not to disturb the pellet and lose cells.
8. Resuspend the cells in a maximum of 60 μL of Annexin Binding Buffer; otherwise, cells will be too diluted to analyze.
9. During the gating of single events and when focusing to gate out the material and nonfocused cells, always observe the cells on the borders of a gate to make sure that the highest accuracy is achieved.
10. With the increasing concentration of the material used for the treatment, the contrast and focus properties of the cells might

change. It is thus allowed to readjust gating in the bivariate plots, by looking at the Imaging Gallery, aiming to exclude the material and nonfocused cells from the analysis.

11. Higher accuracy of the cytotoxicity assessment was achieved using imaging flow cytometry compared to the flow cytometry (FACS Verse instrument) under the same conditions and with the same sample preparation procedures, especially after the treatment with the highest concentration of the L-GO material due to the possibility to observe the events included in the analysis and exclude aggregated material from the analysis (Fig. 5).

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