Project: VIGO

EMPA
Materials Science & Technology

Flow cytometric Annexin V/Propidium Iodide measurement in A549 cells

Detecting apoptosis and necrosis in A549 cells

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DOCUMENT HISTORY

Effective Date	Date Revision Required	Supersedes
15.02.2014	DD/MM/YYYY	DD/MM/YYYY

Version	Approval Date	Description of the Change		Author / Changed by
1.0	DD/MM/YYYY	All	Initial Document	Cordula Hirsch

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1 Introduction

Generally two modes of cell death can be distinguished in multicellular organisms: apoptosis or programmed cell death and necrosis, a form of traumatic cell death.

Apoptosis plays a regulatory function during development and in tissue homeostasis. The “suicide” of cells is activated and controlled by an intracellular death program. It results in characteristic morphological changes as for example blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation. One early event in the signaling cascade includes alterations in the organization of phospholipids in the plasma membrane of most cells. Phosphatidylserine (PS) is located on the cytoplasmic side of the cell membrane in healthy cells. In early apoptotic cells however, PS appears on the extracellular surface of the membrane. PS exposure is recognized by phagocytes *in vivo* which engulf and thereby remove apoptotic cells (or cell fragments, called apoptotic bodies). This coordinated process prevents inflammatory reactions.

In contrast necrosis (from the Greek “the act of killing”) results from acute cellular injury caused by external factors (e.g. toxins). It is characterized by the loss of cell membrane integrity and an uncontrolled release of cytoplasmic components which initiate inflammatory reactions in the surrounding tissue.

In vitro externalized PS can be detected by Annexin V. In the presence of calcium, this anticoagulant binds to PS with high affinity thereby labeling apoptotic cells. Propidium Iodide (PI) intercalates into DNA (and to some extend also RNA) and is membrane impermeable. Therefore it is usually excluded from viable cells and can be used to identify necrotic cells. Bound to nucleic acids the fluorescence excitation maximum of PI is at 535 nm and its emission maximum at 617 nm.

2 Principle of the Method

A fluorescein isothiocyanate (FITC) conjugated form of Annexin V is used to detect PS exposing cells thereby marking apoptotic cells. However, due to membrane disintegration during necrosis, Annexin V will also bind to intracellularly located PS in necrotic cells. Therefore the cell impermeable dye PI is added to distinguish apoptotic and necrotic cells.

- **Viable** cells will remain unstained (no PS exposure – no Annexin V labeling; no membrane - disintegration – no PI in contact with nucleic acids).
- **Apoptotic** cells will stain positive for **Annexin V** (PS exposure but no membrane disintegration).
- **Necrotic** cells will stain positive for **PI** (membrane disintegration) and to a certain extend also for **Annexin V** (PS detection on the intracellular side of the membrane).

As no phagocytes are present in monocultures *in vitro* final stages of apoptosis involve necrotic-like disintegration of the complete cell. Therefore **late apoptotic** cells will also stain positive for both **Annexin V** and **PI**.

The number of Annexin V, PI and Annexin V/PI double labeled cells is quantified using flow cytometry.

3 Applicability and Limitations

To properly adjust the settings of the flow cytometer appropriate positive controls are necessary that reliably induce apoptosis and necrosis, respectively. The stimuli that induce either apoptosis or necrosis will vary from one cell type to the other. Here we describe the assessment of apoptosis and necrosis specifically for A549 cells.

Another issue is time. As mentioned above late stages of apoptosis will result in necrosis-like membrane disintegration *in vitro*. Therefore it is important to elucidate a suitable timeframe for the experimental setup. Apoptotic events might be missed when only very long incubation times are chosen. Accordingly, we assess three different time points here: 3 h for very early events, 24 h as an interim measurement and 72 h for very late events.

Nanomaterials (NMs) are likely to interfere in fluorimetric measurements. Interference considerations for flow cytometric analysis are challenging. Suggestions for an experimental setup are given in the SOP "NM interference in the flow cytometric Annexin V/PI measurement".

Here we describe only the usage of the Annexin V/PI labeling solutions from BD Pharmingen and a Partec CyFlow Space flow cytometer. If any other kits, staining solutions or flow cytometers are used please refer to the manufacturer's descriptions.

This SOP does not give an introduction into flow cytometry per se. Therefore, the operator of this assay has to be well trained in flow cytometry.

4 Related Documents

Table 1: Documents needed to proceed according to this SOP and additional NM-related interference control protocols.

Document ID	Document Title
cell culture_A549	*Culturing A549 cells*
V_AnnexinV/PI_ interference	*NM interference in the flow cytometric Annexin V/Propidium Iodide measurement*
M_NM suspension_metal oxides	*Suspending and diluting Nanomaterials – Metal oxides and NM purchased as monodisperse suspensions*
M_NM suspension_ carbon based	*Suspending and diluting Nanomaterials – Carbon based nanomaterials*

5 Equipment and Reagents

5.1 Equipment

- 6-well cell culture plates
- Centrifuge (for cell pelleting; able to run 15 ml as well as 50 ml tubes at 200 x g)
- Conical tubes (15 ml and 50 ml; polypropylene or polystyrene; e.g. from Falcon)
- Flow cytometer (e.g. CyFlow Space from Partec)
- Hemocytometer
- Laminar flow cabinet (biological hazard standard)
- Light microscope (for cell counting and cell observation)
- Microreaction tubes (1.5 ml; e.g. from Eppendorf)
- Round base tubes (3.5 ml suitable for flow cytometry; polystyrene; e.g. from Sarstedt)
- Vortex®

5.2 Reagents

For cell culturing:

- Fetal Calf Serum (FCS)
- L-glutamine
- Neomycin[1)]
- Penicillin[1)]
- Phosphate buffered saline (PBS)
- Roswell Park Memorial Institute medium (RPMI-1640)
- Streptomycin[1)]
- Trypsin-EDTA (0.05%)

[1)] bought as a 100x concentrated mixture of Penicillin, Streptomycin and Neomycin (PSN) e.g. from Gibco.

Additionally necessary to dilute carbon based NM:

- 10x concentrated RPMI-1640
- Sodium bicarbonate solution, 7.5% ($NaHCO_3$) *[CAS-number: 144-55-8]*

For Annexin V-PI labeling:

- 10x Annexin V binding buffer *[BD Pharmingen; #556454]*
- Annexin V-FITC *[BD Pharmingen; #556419]*
- Cadmium sulfate 8/3-hydrate (3 $CdSO_4 \cdot 8H_2O$) *[CAS number: 7790-84-3]*
  Note: Toxic! Handle with special care!
- Dimethyl sulfoxide (DMSO) *[CAS number: 67-68-5]*
- Propidium Iodide (PI) *[BD Pharmingen; #556463]*
  Note: Potentially mutagenic! Handle with special care!
- Staurosporine *[CAS number: 62996-74-1]*

5.3 Reagent Preparation

5.3.1 Complete cell culture medium

Basic medium:

- RPMI-1640

supplemented with:

- 10% FCS
- 1x PSN, which results in final concentrations of:
  - 50 µg/ml Penicillin
  - 50 µg/ml Streptomycin
  - 100 µg/ml Neomycin
- 0.2 mg/ml L-glutamine

5.3.2 Cadmium sulfate

Prepare a 1 M stock solution in ddH_2O. Can be stored at 4°C for several months.

- Dissolve 2.57 g $CdSO_4 \cdot 8/3\ H_2O$ in 10 ml ddH_2O.

5.3.3 Staurosporine

Prepare a 1 mM stock solution in DMSO. Prepare single use aliquots that can be stored at -20°C for several months.

- Dissolve 0.467 mg staurosporine in 1 ml DMSO.

5.3.4 1x Annexin V binding buffer

Dilute the 10x concentrated stock (supplied by the manufacturer) to a 1x concentrated working solution in ddH_2O. Prepare freshly for each experiment and store on ice until usage.

- Mix 18 ml of ddH_2O with 2 ml of the 10x concentrated stock.

6 Procedure

6.1 General remarks

To avoid contact inhibition of A549 cells during assay performance different cell numbers are seeded on experimental start. Appropriate concentrations and incubation times for the two positive controls ($CdSO_4$ to induce necrosis and Staurosporine to induce apoptosis) were established in pilot tests and will have to be optimized for any other cell type used. The combination of the two controls is utilized to assess assay performance in general and to adjust the settings of the flow cytometer properly.

Find more details in chapter 6.5 “Cell harvest and flow cytometric analysis”.

6.2 Flow chart

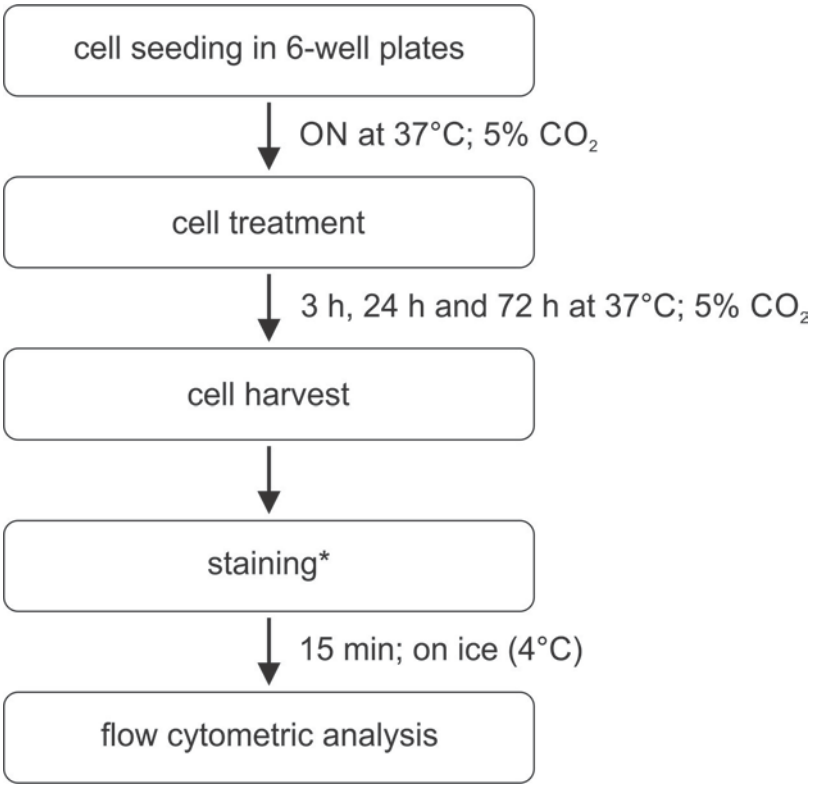

Figure 1: Brief outline of the workflow: from cell seeding to analysis.
*To adjust the two channels (FL1-green: Annexin V-FITC and FL2-red: PI) of the flow cytometer and to detect and compensate for fluorescence crosstalk the following control staining of dying cells is necessary:
- unstained cells
- Annexin V single labeling
- PI single labeling
- Annexin V/PI double labeling

6.3 Cell seeding

6.3.1 Cell culture

A549 cells are grown in T75 cell culture flasks in a total volume of 20 ml of complete cell culture medium. They are kept at 37°C, 5% CO_2 in humidified air in an incubator (standard growth conditions according to SOP "Culturing A549 cells").

6.3.2 Cell seeding into 6-well plates

- One day prior to experimental start harvest and count cells as described in SOP "Culturing A549 cells".
- Seed cells in 2.5 ml complete cell culture medium per well into 6-well cell culture plates as depicted for one time point in Figure 2.
- As A549 cells proliferate, different cell numbers are seeded for the different time points of analysis:
    - 3 h: 2.5×10^5 cells per well in 2.5 ml complete cell culture medium. For the 15 wells of this time point 4×10^6 cells are suspended in 40 ml complete cell culture medium.
    - 24 h: 1×10^5 cells per well in 2.5 ml complete cell culture medium. For the 15 wells of this time point 1.6×10^6 cells are suspended in 40 ml complete cell culture medium.
    - 72 h: 2×10^4 cells per well in 2.5 ml complete cell culture medium. For the 15 wells of this time point 3.2×10^5 cells are suspended in 40 ml complete cell culture medium.
- Using a 5 ml pipette 2.5 ml of the respective cell suspensions are distributed into each of the green wells depicted in Figure 2.
- Cells are kept in a humidified incubator at standard growth conditions overnight (ON).

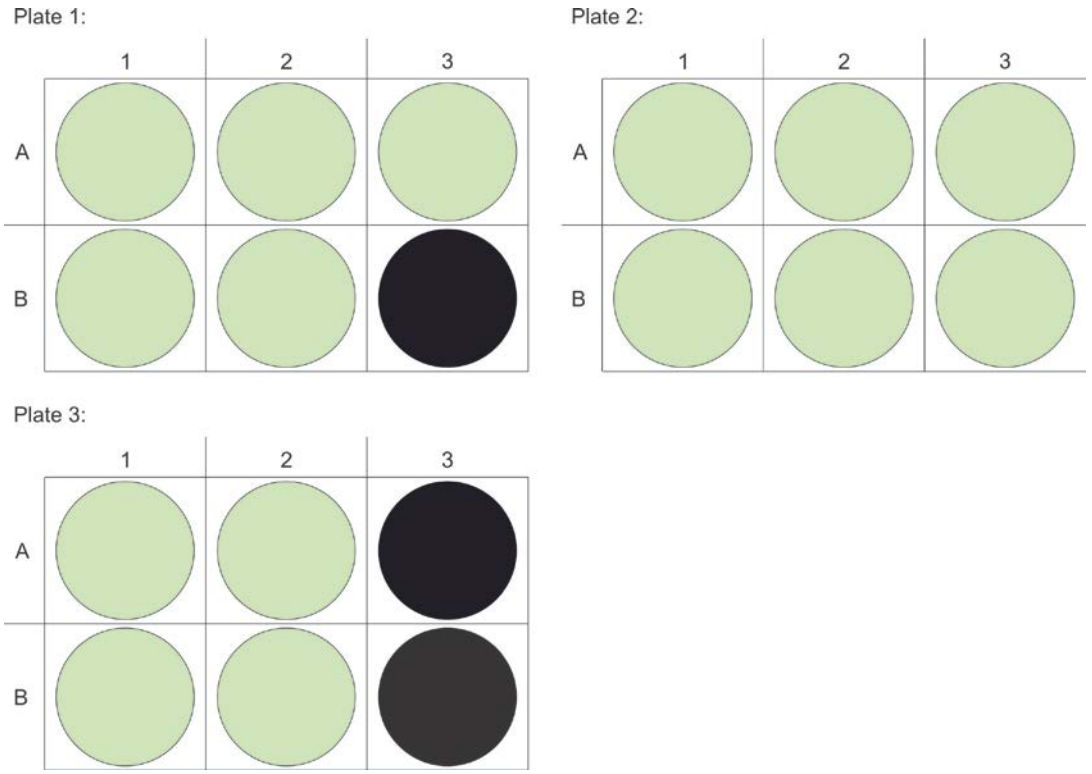

Figure 2: Cell seeding into 6-well plates. Depicted are 3 plates (15 wells in green) necessary to analyze one time point. Multiply for each additional time point. **Note:** Cell numbers per well are different for the three time points (compare main text). Black wells remain empty.

6.4 Cell treatment

6.4.1 Dilution of nanomaterials

For this SOP we distinguish two types of nanomaterials (NM) according to their solvent, suspension properties and highest concentrations used in the assay. See also respective related documents (3).

(1) Metal oxide NM, Polystyrene beads and all NM delivered as monodisperse suspensions by the supplier: solvent either determined by the supplier or ddH_2O; sub-diluted in ddH_2O; highest concentration in assay 100 µg/ml
(2) Carbon based NM: suspended and sub-diluted in 160 ppm Pluronic F-127; highest concentration in assay 80 µg/ml

Volumes given in the following dilution schemes are enough for all three time points to be analyzed in 6-well plates as shown in Figure 2.

Note: "Mixing" in the context of diluting NMs means, the solvent containing tube is put on a continuously shaking Vortex® and the previous sub-dilution (or stock suspension, respectively) is put dropwise into the shaking solvent. The resulting suspension stays on the Vortex® for additional 3 seconds before proceeding with the next sub-dilution.

(1) Metal oxide NM:

Prepare serial sub-dilutions of the stock suspension (1 mg/ml) in ddH_2O:

- Label eight conical tubes (15 ml total volume) with 1 to 8 (relates to steps 1-8 below).
- Add 1.5 ml ddH_2O to tubes 2 to 8.

1. 3 ml NM stock suspension in ddH_2O → 1 mg/ml (1)
2. 1.5 ml of 1 mg/ml stock suspension are mixed with 1 ml of ddH_2O → 500 µg/ml (2)
3. 1.5 ml of 500 µg/ml (2) are mixed with 1.5 ml ddH_2O → 250 µg/ml (3)
4. 1.5 ml of 250 µg/ml (3) are mixed with 1.5 ml ddH_2O → 125 µg/ml (4)
5. 1.5 ml of 125 µg/ml (4) are mixed with 1.5 ml ddH_2O → 62.5 µg/ml (5)
6. 1.5 ml of 62.5 µg/ml (5) are mixed with 1.5 ml ddH_2O → 31.3 µg/ml (6)
7. 1.5 ml of 31.3 µg/ml (6) are mixed with 1.5 ml ddH_2O → 15.6 µg/ml (7)
8. 1.5 ml ddH_2O → solvent control (8)

Preparation of final dilutions:

- Label eight conical tubes (15 ml total volume) as follows:
    1. 100 µg/ml
    2. 50 µg/ml
    3. 25 µg/ml
    4. 12.5 µg/ml
    5. 6.25 µg/ml
    6. 3.13 µg/ml
    7. 1.56 µg/ml
    8. ddH_2O: Solvent control
- Add 9 ml complete cell culture medium to each tube.
- Mix on the Vortex with 1 ml of the respective NM sub-dilutions or the solvent (ddH_2O):
    1. 1 ml of the stock suspension (1 mg/ml) are mixed with 9 ml medium → 100 µg/ml (1)
    2. 1 ml of the 500 µg/ml sub-dilution are mixed with 9 ml medium → 50 µg/ml (2)
    3. 1 ml of the 250 µg/ml sub-dilution are mixed with 9 ml medium → 25 µg/ml (3)
    4. 1 ml of the 125 µg/ml sub-dilution are mixed with 9 ml medium → 12.5 µg/ml (4)
    5. 1 ml of the 62.5 µg/ml sub-dilution are mixed with 9 ml medium → 6.25 µg/ml (5)
    6. 1 ml of the 31.3 µg/ml sub-dilution are mixed with 9 ml medium → 3.13 µg/ml (6)
    7. 1 ml of the 15.6 µg/ml sub-dilution are mixed with 9 ml medium → 1.56 µg/ml (7)
    8. 1 ml of ddH_2O (solvent) are mixed with 9 ml medium → solvent control (8)

(2) Carbon based NM:

Prepare serial sub-dilutions of the stock suspension (500 µg/ml) in 160 ppm Pluronic F-127:

- Label eight conical tubes (15 ml total volume) with 1 to 8 (relates to steps 1-8 below).
- Add 2 ml 160 ppm Pluronic F-127 to tubes 2 to 8.

1. 4 ml NM stock suspension in Pluronic F-127 → 500 µg/ml (1)
2. 2 ml of 500 µg/ml stock suspension are mixed with 2 ml of Pluronic F-127 → 250 µg/ml (2)
3. 2 ml of 250 µg/ml (2) are mixed with 2 ml Pluronic F-127 → 125 µg/ml (3)
4. 2 ml of 125 µg/ml (3) are mixed with 2 ml Pluronic F-127 → 62.5 µg/ml (4)
5. 2 ml of 62.5 µg/ml (4) are mixed with 2 ml Pluronic F-127 → 31.3 µg/ml (5)
6. 2 ml of 31.3 µg/ml (5) are mixed with 2 ml Pluronic F-127 → 15.6 µg/ml (6)
7. 2 ml of 15.6 µg/ml (6) are mixed with 2 ml Pluronic F-127 → 7.8 µg/ml (7)
8. 2 ml Pluronic F-127 → solvent control (8)

Preparation of final dilutions:

- Prepare the appropriate dilution of a 10x concentrated medium stock as follows. This mixture (A) is used in all following steps for the preparation of the final NM concentrations. Mixing NM sub-dilutions with (A) will result in 1x concentrated medium containing the correct concentrations of all supplements and the respective NM concentrations.

Reagent	**Volume**	
10x RPMI	7	ml
100x PSN	700	µl
100x L-Glutamine	700	µl
7.5% $NaHCO_3$	1.87	ml
100% FCS	7	ml
ddH_2O	41	ml

- Label eight conical tubes (15 ml total volume) as follows:
    1. 80 µg/ml
    2. 40 µg/ml
    3. 20 µg/ml
    4. 10 µg/ml
    5. 5 µg/ml
    6. 2.5 µg/ml
    7. 1.25 µg/ml
    8. Pluronic F-127: Solvent control
- Add 7.4 ml (A) to each tube. Then mix on the Vortex® with 1.6 ml of the respective NM sub-dilutions or the solvent (160 ppm Pluronic F-127):
    1. 1.6 ml of the stock suspension (500 µg/ml) are mixed with 7.4 ml medium (A) → 80 µg/ml (1)
    2. 1.6 ml of the 250 µg/ml sub-dilution are mixed with 7.4 ml medium (A) → 40 µg/ml (2)
    3. 1.6 ml of the 125 µg/ml sub-dilution are mixed with 7.4 ml medium (A) → 20 µg/ml (3)
    4. 1.6 ml of the 62.5 µg/ml sub-dilution are mixed with 7.4 ml medium (A) → 10 µg/ml (4)

5. 1.6 ml of the 31.3 µg/ml sub-dilution are mixed with 7.4 ml medium (A)
   → 5 µg/ml (5)
6. 1.6 ml of the 15.6 µg/ml sub-dilution are mixed with 7.4 ml medium (A)
   → 2.5 µg/ml (6)
7. 1.6 ml of the 7.8 µg/ml sub-dilution are mixed with 7.4 ml medium (A)
   → 1.25 µg/ml (7)
8. 1.6 ml of 160 ppm Pluronic F-127 (solvent) are mixed with 7.4 ml medium (A)
   → solvent control (8)

6.4.2 Dilution of $CdSO_4$ (inducer of necrosis)

This stimulus is added **three hours before cell harvest**. Therefore this dilution has to be prepared freshly for each time point. The volumes given here are enough for one time point only. To obtain a **5 mM working concentration**:

- Mix 7 ml complete cell culture medium with 35 µl of the 1 M $CdSO_4$ stock solution.

6.4.3 Dilution of Staurosporine (inducer of apoptosis)

This stimulus is added **four hours before cell harvest**. Therefore this dilution has to be prepared freshly for each time point. The volumes given here are enough for one time point only. To obtain a **4 µM working concentration:**

- Mix 7 ml complete cell culture medium with 28 µl of the 1 mM Staurosporine stock solution.

6.4.4 Dilution of DMSO (solvent control for Staurosporine)

Staurosporine is dissolved in DMSO. Therefore cells are incubated for four hours with the same volume of DMSO as the solvent control.

- Mix 7 ml complete cell culture medium with 28 µl of DMSO.

6.4.5 Application of stimuli

Each of the three time points receive the NM dilutions (as well as the NM solvent) at the same time one day after cell seeding.

Note: All NM dilutions have to be vortexed directly before application to the cells.

As the inducers for necrosis ($CdSO_4$) and apoptosis (Staurosporine) are applied 3 and 4 hours before cell harvest, respectively, their application times will differ for each of the three time points. Plate layouts are the same for all time points. Distribution of the samples as shown in Figure 3 assures that NM treated and untreated samples can remain undisturbed for the whole incubation time. Only control samples (all on plate 1) are taken out of the incubator at respective time points to receive stimuli. To have enough cells to adjust the setup of the flow cytometer, 2 wells of these control samples are necessary.

For the **3 h time point** Staurosporine and its solvent DMSO are added first. One hour later all other stimuli ($CdSO_4$, NMs, solvents) are added and another 3 hours later cells are harvested.

- Remove complete cell culture medium from wells A2, B2 and A3 on plate 1 of the 3 h time point (see Figure 3).

- Add 2.5 ml per well of complete cell culture medium containing 4 µM Staurosporine to wells A2 and B3.
- Add 2.5 ml of complete cell culture medium containing DMSO to well A3.
- Incubate for 1 h under standard growth conditions.
- Remove complete cell culture medium from wells A1 and B1 on plate 1.
- Add 2.5 ml per well of complete cell culture medium containing 5 mM $CdSO_4$.
- Remove complete cell culture medium from all remaining wells on plates 2 and 3 from **all time points**.
- Add 2.5 ml complete cell culture medium containing the corresponding NM concentrations or the NM solvent control according to the pipetting scheme shown in Figure 3.
- Add 2.5 ml complete cell culture medium per well for untreated samples (plate 3 wells B1 and B2).
- Incubate for appropriate time points under standard growth conditions.

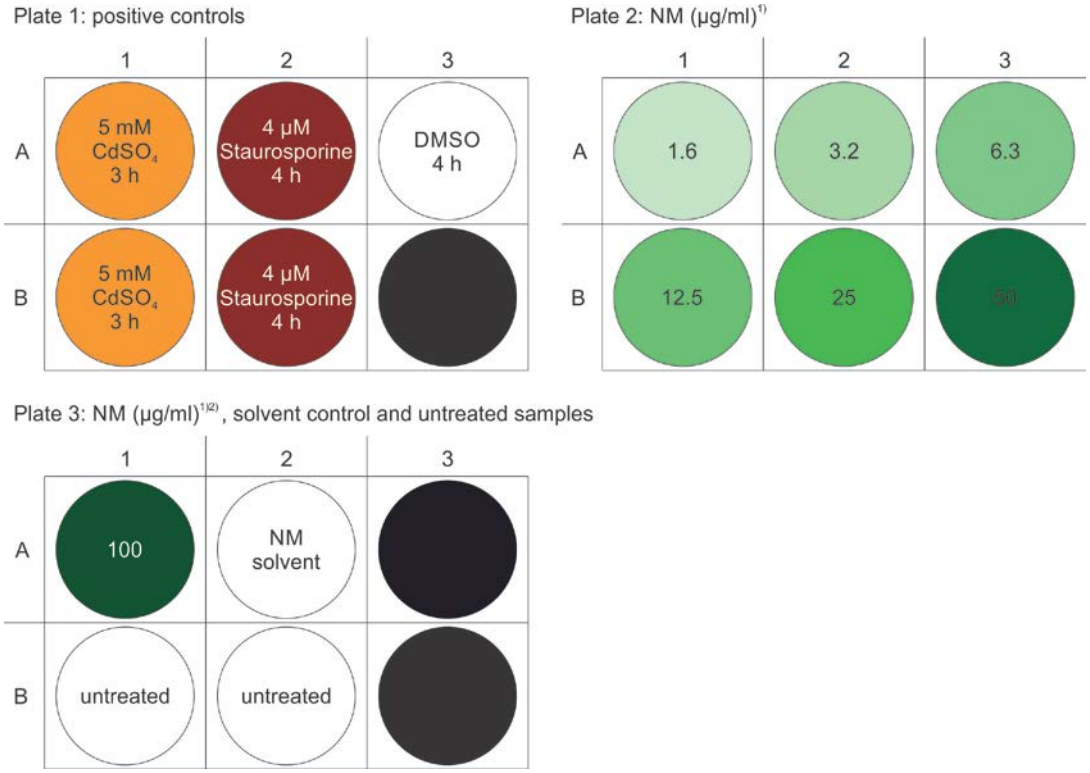

Figure 3: Application of stimuli. NMs as well as the solvent are applied at the same time in 2.5 ml complete cell culture medium per well. Untreated samples (plate 3 wells B1 and B2) receive complete cell culture medium only. Black wells remain empty. Cells are incubated at standard growth conditions for appropriate time points (3 h, 24 h, 72 h).
[1)] NM concentrations given here refer to metal oxide NMs. Carbon based NM concentrations are detailed in the text.
Note: Apoptosis (Staurosporine) and necrosis ($CdSO_4$) inducers are applied 4 h and 3 h before cell harvest, respectively. Thus their application time will differ for each of the three time points. For details see main text.

6.5 Cell harvest and flow cytometric analysis

6.5.1 To be set before cell harvest

Note: To assure that all cells – even dead cells floating already in the supernatant – enter the final flow cytometric analysis all supernatants are collected. Therefore prepare a set of 12 conical tubes (15 ml). One for each treatment condition labeled as follows:

1. $CdSO_4$
2. Staurosporine
3. Untreated

Note: These three samples will contain twice as much volume as the others as the content of the 2 6-wells will be pooled.

4. DMSO
5. 1.6 µg/ml NM
6. 3.2 µg/ml NM
7. 6.3 µg/ml NM
8. 12.5 µg/ml NM
9. 25 µg/ml NM
10. 50 µg/ml NM
11. 100 µg/ml NM
12. NM solvent

In addition:

- Prepare an **ice box** to cool down samples after harvest and for the staining process.
- Make sure to have appropriate amount of **1x Annexin V binding buffer** ready on ice. For one time point 20 ml will be enough.

- Dilution of dyes:
  Staining is performed in a total volume of 100 µl per sample. Volume ratios for **ONE** sample are given in Table 2.

Table 2: Dilution scheme for Annexin V and PI for one sample.

	a) Annexin V-FITC single labeling	**b) PI single labeling**	**c) Double labeling**
Annexin V-FITC	5 µl	0 µl	5 µl
PI	0 µl	5 µl	5 µl
1x Annexin V binding buffer	95 µl	95 µl	90 µl
Total volume	100 µl	100 µl	100 µl

Volumes given below are enough for **one time point:**

a) Annexin V:
   Mix 475 µl 1x Annexin V binding buffer with 25 µl Annexin V stock solution.
b) PI:
   Mix 475 µl 1x Annexin V binding buffer with 25 µl PI stock solution.
c) AnnexinV/PI:
   Mix 1260 µl 1x Annexin V binding buffer with 70 µl Annexin V stock solution and 70 µl PI stock solution.

6.5.2 Cell harvest

- After appropriate time points (3 h, 24 h, 72 h) transfer complete cell culture medium of all samples into the respective labeled conical tubes.
- Wash cells twice with 1 ml pre-warmed (37°C) PBS. Transfer PBS to the same corresponding conical tube as the complete cell culture medium before.
- To detach cells add 0.5 ml Trypsin-EDTA per well and incubate for approximately 5 min at 37°C.
- Add 1 ml complete cell culture medium to inhibit Trypsin and stop the detachment reaction.
- Transfer cell suspension also to the corresponding conical tubes.
- Spin cells down at 200 x g for 5 min.
- Remove supernatant carefully.
  Note: Avoid losing cells; rather leave some liquid (~50 µl) on top of the cell pellet.
- Put cell pellets on ice.

6.5.3 Staining

6.5.3.1 Preparation of control samples

- Resuspend the cell pellets of
  1. $CdSO_4$
  2. Staurosporine
  3. Untreated

  samples in 400 µl 1x binding buffer.

- From samples 1 and 2 prepare 4 aliquots à 100 µl in 1.5 ml microreaction tubes labeled with the respective treatment condition (*"$CdSO_4$"* or *"Staurosporine"*) and the following additions:
  a) Annexin V
  b) PI
  c) Annexin V/PI
  d) Unstained
- From sample 3 prepare 2 aliquots à 200 µl in 1.5 ml microreaction tubes labeled with the respective treatment condition (*"untreated"*) and the following additions:
  c) Annexin V/PI
  d) Unstained
- Spin down again at 200 x g for 5 min.

- Resuspend pellets in 100 µl of the respective staining solutions (a-c described above) or 100 µl 1x binding buffer only (unstained).
- Incubate on ice for 15 min.

6.5.3.2 Preparation of all other samples

- Resuspend cell pellets (on ice in 15 ml conical tubes) in 100 µl per sample of the AnnexinV/PI dilution (c: as described above)
- Incubate on ice for 15 min.

6.5.4 Flow cytometric analysis

- Record the following parameters in 4 graphs (as shown in Figure 4):
    a) Dot plot 1: Forward scatter (FSC) vs. Side scatter (SSC)
    b) Histogram 1: FL1-FITC (Annexin V-FITC) vs. cell counts
    c) Histogram 2: FL2-PI (PI) vs. cell counts
    d) Dot plot 2: FL1-FITC vs. FL2-PI
- FSC and SSC are recorded linearly while both fluorescence channels (FL1 and FL2) are recorded on a log scale.

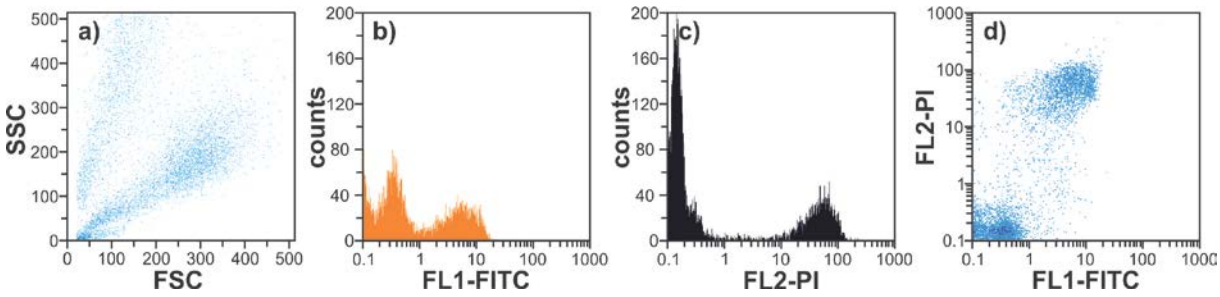

Figure 4: Data are recorded in four plots. a) Dot plot recording forward and side scatter to assess cell size and granularity, respectively. b) Histogram FL1-FITC vs. cell counts, detecting the number of Annexin V-FITC positive cells. c) Histogram FL2-PI vs. cell counts, detecting the number of PI positive cells. d) Dot plot recording FL1 and FL2 to assess both colors in one graph and with that single as well as double positive cells for either dye. Shown is an example of $CdSO_4$ treated A549 cells.

- Use the control samples to adjust the instrument settings of the flow cytometer:
    - Untreated cells – unstained: used to adjust the cloud of the cell population in the FSC/SSC plot as well as to define the boundaries of quadrant Q3 in the FL1/FL2 plot, which contains unstained healthy cells (see Figure 5).
    - Dying cells ($CdSO_4$ and Staurosporine treated samples) – unstained: check if and how cell population changes in the FSC/SSC plot and FL1/FL2 plot. Make minor adjustments (if needed) to detect both populations (dead and alive) with the same settings.
    - Dying cells ($CdSO_4$ and Staurosporine treated samples) – Annexin V single labeling: used to set the boundaries of quadrant Q4 in the FL1/FL2 plot which contains Annexin V positive/PI negative apoptotic cells.
      Additionally used to check for fluorescence crosstalk and to set up compensation.
    - Dying cells ($CdSO_4$ and Staurosporine treated samples) – PI single labeling: used to set the boundaries of quadrant Q1 in the FL1/FL2 plot which contains Annexin V

negative/PI positive necrotic cells.
Additionally used to check for fluorescence crosstalk and to set up compensation.

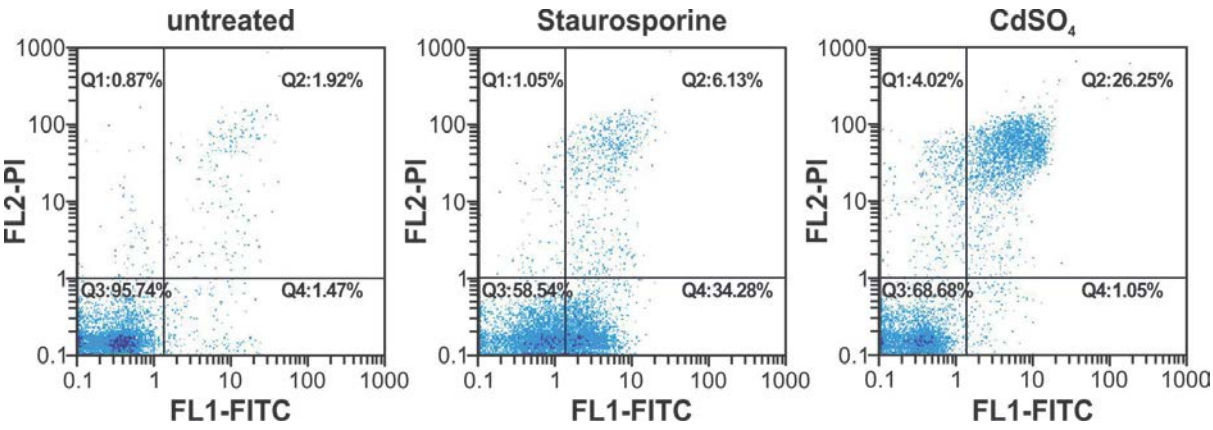

Figure 5: Example of Annexin V-FITC/PI double labeled A549 cells. Comparing untreated, Staurosporine and $CdSO_4$ treated AnnexinV/PI double labeled cells illustrates the different modes of cell death and how single and double labeled cells distribute in the dot plot.

- Record Annexin V/PI double labeling data of 10 000 cells per treatment condition. Therefore dilute 30 µl of the stained samples (total volume of 100 µl) in 1 ml 1x Annexin V binding buffer in round base flow cytometry tubes.
  Note: In samples of dying cells 30 µl of the cell suspension might not be enough to reach the 10 000 cells. Adjust the volume accordingly.

6.6 Data evaluation

Data are presented in a stacked bar chart as shown in Figure 6. Annexin V positive/PI negative cells are apoptotic and appear in quadrant Q4 in the FL1/FL2 plot. Quadrants Q1 and Q2 contain Annexin V negative/PI positive as well as double positive cells and are summarized as the late apoptotic/necrotic population.

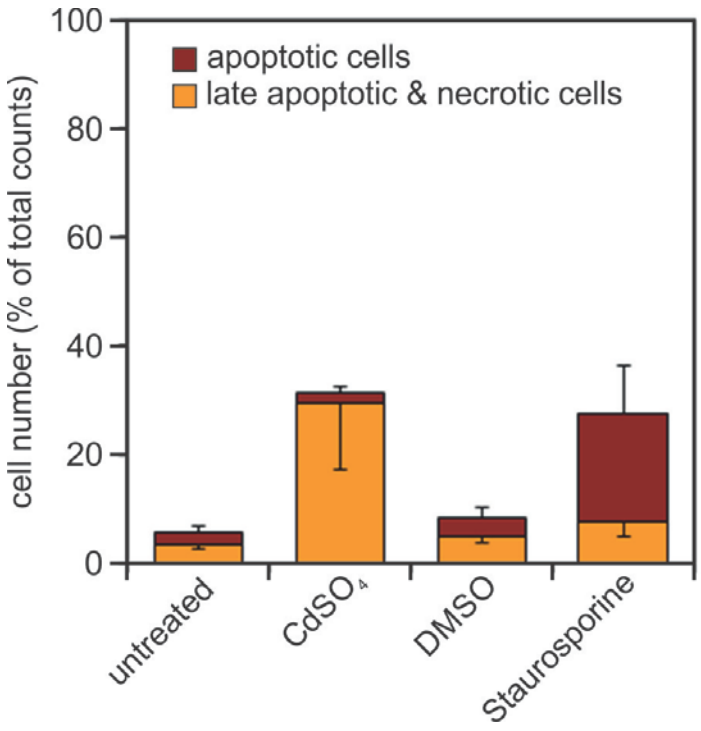

Figure 6: Example of data presentation in a stacked bar chart. A549 cells were either left untreated or received 5 mM $CdSO_4$ for 3 h or 4 µM Staurosporine for 4 h. DMSO served as the solvent control for Staurosporine and was also applied for 4 h. Cells were stained with Annexin V-FITC and PI. Shown are the mean values of seven independent experiments and their standard deviations.

7 Quality Control, Quality Assurance, Acceptance Criteria

Pay attention to the following parameters:

- $CdSO_4$ treatment should result in 15% to 25% late apoptotic/necrotic cells while the fraction of apoptotic cells should not increase.
- Staurosporine treatment should result in 10% to 20% apoptotic cells and only a minor increase in late apoptotic/necrotic cells.
- Untreated samples should not contain more than 5% to 10% dead cells in total (apoptotic and necrotic).

8 Health and Safety Warnings, Cautions and Waste Treatment

Cell seeding has to be carried out under sterile conditions in a laminar flow cabinet (biological hazard standard). For this only sterile equipment must be used and operators should wear laboratory coat and gloves (according to laboratory internal standards).

Discard all materials used to handle cells (including remaining cells themselves) according to the appropriate procedure for special biological waste (i.e. by autoclaving).

Handle the following chemicals with special care. **Wear suitable protective clothing** (especially **gloves**, lab coat, respiratory protection (if handling the powder)).

- **PI** may be irritating to the skin, eyes and respiratory organs. It may be harmful if swallowed, inhaled or absorbed through skin. It is a possible mutagen and may cause heritable genetic damage.

- **$CdSO_4$** is toxic if swallowed, very toxic by inhalation and may even cause cancer. It is irritating to eyes and skin, may cause heritable genetic damage, may impair fertility and may cause harm to the unborn child.

9 Abbreviations

ddH_2O	double-distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	constant of gravitation
NM	nanomaterial
ON	overnight
PBS	phosphate buffered saline
PI	Propidium Iodide
ppm	parts per million
PS	Phosphatidyl serine
PSN	Penicillin, Streptomycin, Neomycin
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SSC	side scatter

10 References