Project: VIGO

EMPA
Materials Science & Technology

# NM interference in the flow cytometric Annexin V/Propidium Iodide measurement

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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 |

# Table of Content

# 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.

How nanomaterials (NM) might interfere in the assessment of apoptotic and necrotic cells by Annexin V/PI labeling will be discussed.

# 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.

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| NMs might agglomerate in cell culture medium. Large agglomerates might be detectable by flow cytometry and might cause false results. Therefore we check in a cell free control setup the appearance of NMs. Even if no NMs (or their agglomerates) are detectable, the presence of NMs (or their agglomerates) might change the appearance of healthy cells (e.g. granularity, size or fluorescence) without influencing their viability. To address this issue we developed a set of controls bringing together healthy cells and NMs shortly before flow cytometric analysis. |

# 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.

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| NM interference considerations for flow cytometric analysis are challenging. We do not claim to fully cover all possible interference reactions. The described setup is supposed to give an idea of interference testing only. |

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 of 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 grounded in flow cytometry.

# 4 Related Documents

**Table 1:** Documents needed to proceed according to this SOP.

| Document ID | Document Title |
|---|---|
| cell culture_A549 | *Culturing A549 cells* |
| 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* |
| V_AnnexinV-PI_A549 | *Flow cytometric Annexin V/Propidium Iodide measurement in A549 cells* |

# 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)
- T75 cell culture flasks
- 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.

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 54 ml of $ddH_2O$ with 6 ml of the 10x concentrated stock.

# 6 Procedure

## 6.1 General remarks

Apoptosis and necrosis are induced through Staurosporine and $CdSO_4$ treatment of A549 cells, respectively. In the context of NM interference testing these control treatments are used to adjust the settings of the flow cytometer, only. Therefore cells are seeded in 6-well plates as described shortly below (6.3.2).

A considerable amount of a homogenous untreated cell suspension is needed to assess the influence of NMs on the staining process and on the cells in general. Therefore cells are grown in T75 flasks.

Finally, NM dilutions in different solvents (in the absence of cells!) are measured flow cytometrically with exactly the same settings adjusted before. Thereby NM agglomerates can be detected.

## 6.2 Flow chart: interference considerations in the presence of cells

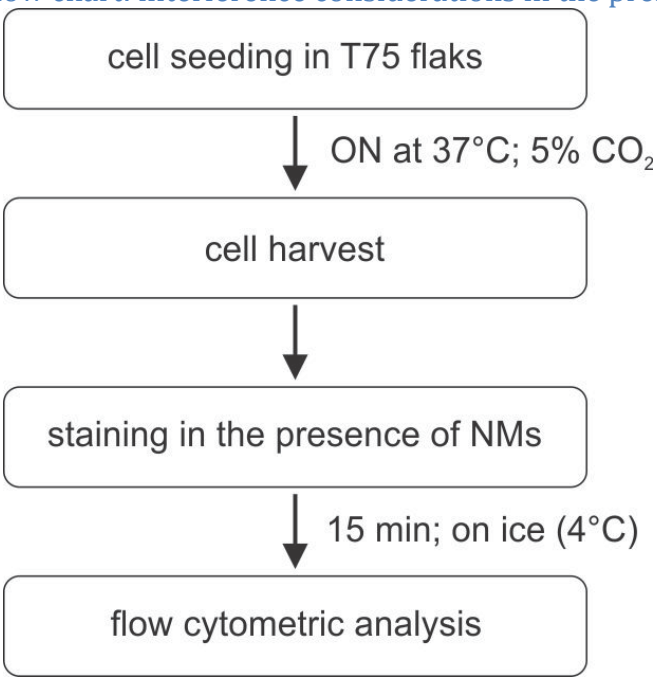


**Figure 1: Brief outline of the workflow:** from cell seeding to analysis.

## 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 (control samples)

- One day prior to experimental start harvest and count cells as described in SOP "Culturing A549 cells".
- Seed $2.5x10^5$ cells in 2.5 ml complete cell culture medium per well into 6-well cell culture plates as shown in Figure 2.
- To fill the four green wells (as shown in Figure 2) $1.25x10^6$ cells are suspended in 12.5 ml complete cell culture medium ($1x10^5$ cells/ml).
- Using a 5 ml pipette 2.5 ml of the cell suspension are distributed into each of the green wells. Black wells remain empty.
- Cells are kept in a humidified incubator at standard growth conditions overnight (ON).

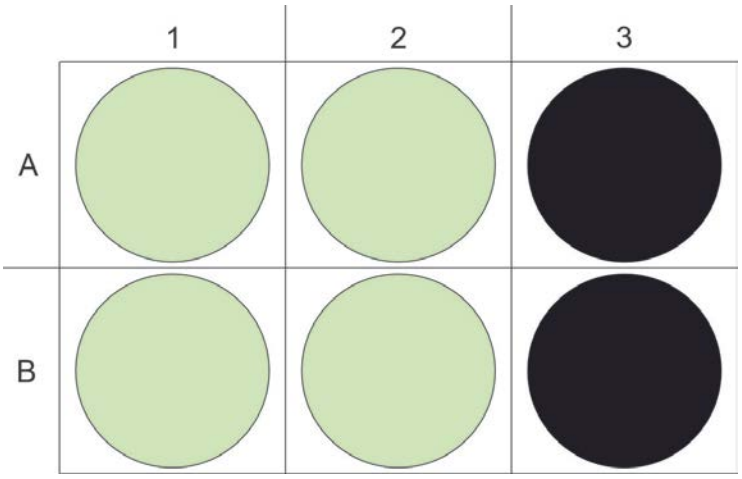


**Figure 2: Cell seeding into 6-well plates.** $2.5x10^5$ cells are seeded in 2.5 ml complete cell culture medium per well. Black wells remain empty.

### 6.3.3 Cell seeding into T75 flasks (untreated samples)

- Two T75 flasks are necessary.
- Seed $2.25x10^6$ cells in 20 ml complete cell culture medium into each of the two T75 flasks. A total volume of 40 ml are needed in which $4.5x10^6$ cells are suspended.
- Cells are kept in a humidified incubator at standard growth conditions ON.

## 6.4 Cell treatment

### 6.4.1 Dilution of $CdSO_4$ (inducer of necrosis)

This stimulus is added **three hours before cell harvest**. 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.

### 6.4.2 Dilution of Staurosporine (inducer of apoptosis)

This stimulus is added **four hours before cell harvest**. To obtain a **4 µM working concentration:**

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

### 6.4.3 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.

### 6.4.4 Application of stimuli

Staurosporine and its solvent DMSO are added first. One hour later $CdSO_4$ is added and another 3 hours later cells are harvested.

- Remove complete cell culture medium from wells A2 and B2 (see Figure 3).
- Add 2.5 ml per well of complete cell culture medium containing 4 µM Staurosporine to wells A2 and B2.
- Incubate for 1 h under standard growth conditions.

- Remove complete cell culture medium from wells A1 and B1.
- Add 2.5 ml per well of complete cell culture medium containing 5 mM $CdSO_4$.
- Incubate for additional three hours under standard growth conditions.

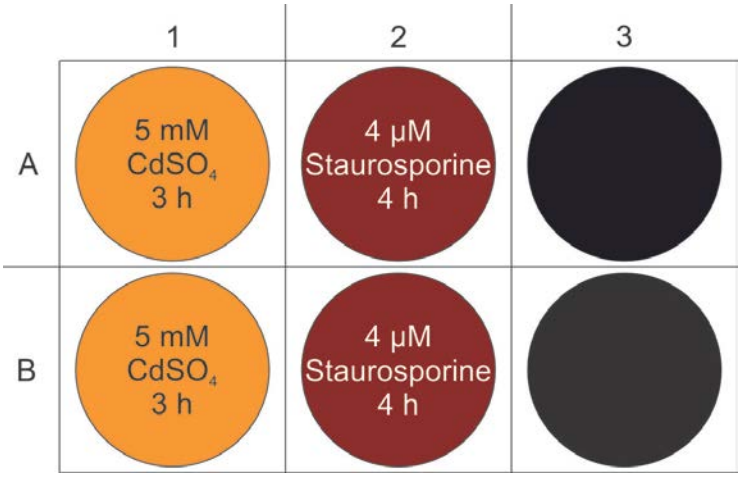


**Figure 3: Application of stimuli.**

**Note:** In the meantime NM dilutions are prepared.

## 6.5 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 the cellular (6.6.3.2) as well as cell free (6.6.3.3) interference control samples.

**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 microreaction tubes (1.5 ml total volume) with 1 to 8 (relates to steps 1-8 below).
- Add 60 µl $ddH_2O$ to tubes 2 to 8.

1. 200 µl NM stock suspension in $ddH_2O$ → 1 mg/ml (1)
2. 60 µl of 1 mg/ml stock suspension are mixed with 60 µl of $ddH_2O$ → 500 µg/ml (2)
3. 60 µl of 500 µg/ml (2) are mixed with 60 µl $ddH_2O$ → 250 µg/ml (3)
4. 60 µl of 250 µg/ml (3) are mixed with 60 µl $ddH_2O$ → 125 µg/ml (4)
5. 60 µl of 125 µg/ml (4) are mixed with 60 µl $ddH_2O$ → 62.5 µg/ml (5)
6. 60 µl of 62.5 µg/ml (5) are mixed with 60 µl $ddH_2O$ → 31.3 µg/ml (6)
7. 60 µl of 31.3 µg/ml (6) are mixed with 60 µl $ddH_2O$ → 15.6 µg/ml (7)
8. 60 µl $ddH_2O$ → solvent control (8)

Final dilutions are prepared directly in the respective staining solutions (with or without cells) as described below (see Figure 4, Figure 5 and Table 3). 10 µl of each NM dilution per 100 µl staining solution are necessary to reach the final concentrations.

**(2) Carbon based NM:**

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

- Label eight microreaction tubes (1.5 ml total volume) with 1 to 8 (relates to steps 1-8 below).
- Add 60 µl 160 ppm Pluronic F-127 to tubes 2 to 8.

1. 400 µl NM stock suspension in Pluronic F-127 → 500 µg/ml (1)
2. 100 µl of 500 µg/ml stock suspension are mixed with 100 µl of Pluronic F-127 → 250 µg/ml (2)
3. 100 µl of 250 µg/ml (2) are mixed with 100 µl Pluronic F-127 → 125 µg/ml (3)
4. 100 µl of 125 µg/ml (3) are mixed with 100 µl Pluronic F-127 → 62.5 µg/ml (4)
5. 100 µl of 62.5 µg/ml (4) are mixed with 100 µl Pluronic F-127 → 31.3 µg/ml (5)
6. 100 µl of 31.3 µg/ml (5) are mixed with 100 µl Pluronic F-127 → 15.6 µg/ml (6)
7. 100 µl of 15.6 µg/ml (6) are mixed with 100 µl Pluronic F-127 → 7.8 µg/ml (7)
8. 100 µl Pluronic F-127 → solvent control (8)

Final dilutions are prepared directly in the respective staining solutions (with or without cells) as described below (see Figure 4, Figure 5 and Table 3). 16 µl of each NM dilution per 100 µl staining solution are necessary to reach the final concentrations.

## 6.6 Cell harvest, staining and flow cytometric analysis

**Note:** For an illustration of the procedure from cell harvest to staining and NM application see Figure 4 and Figure 5.

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 to perform the whole interference experiment (cellular and cell free).

### 6.6.1 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 of control treated samples (6-well plate) are collected. Therefore prepare a set of two conical tubes (15 ml). One for each treatment condition labeled as follows:

1. $CdSO_4$
2. Staurosporine

In addition:

- Prepare a conical tube (50 ml) to harvest the two T75 flasks of untreated cells.

- Prepare an **ice box** to cool down samples after harvest and for the staining process.
- Make sure to have appropriate amount (60 ml) of **1x Annexin V binding buffer** ready on ice.
- Prepare the following dye dilutions (volumes given are for cell free AND cellular controls):
    a) Annexin V:
    Mix 1425 µl 1x Annexin V binding buffer with 75 µl Annexin V stock solution.
    b) PI:
    Mix 1425 µl 1x Annexin V binding buffer with 75 µl PI stock solution.
    c) Annexin V/PI:
    Mix 1350 µl 1x Annexin V binding buffer with 75 µl Annexin V stock solution and 75 µl PI stock solution.

### 6.6.2 Cell harvest

#### *6.6.2.1 Control samples (6-well plate)*

- Transfer complete cell culture medium from $CdSO_4$ and Staurosporine treated cells 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.

#### *6.6.2.2 Untreated samples (T75 flasks)*

- Remove complete cell culture medium from both T75 flasks.
- Wash cells twice with 20 ml pre-warmed (37°C) PBS each.
- To detach cells add 2 ml Trypsin-EDTA per flask and incubate for approximately 5 min at 37°C.
- Add 18 ml complete cell culture medium to inhibit Trypsin and stop the detachment reaction.
- Transfer resulting cell suspensions from both T75 flasks into one conical tube (50 ml).
- The resulting 40 ml cell suspension (20 ml per T75 flask) are divided into 4 aliquots à 10 ml in 4 separate conical tubes (15 ml) labeled as *"untreated"* and the following additions:
  a) Annexin V
  b) PI
  c) Annexin V/PI
  d) Unstained
- 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.

### 6.6.3 Staining

#### *6.6.3.1 Preparation of control samples*

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

  samples in 400 µl 1x binding buffer.

- Prepare 4 aliquots à 100 µl in 1.5 ml microreaction tubes per sample 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
- 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 (d) unstained).
- Incubate on ice for 15 min.

### 6.6.3.2 Preparation of untreated samples

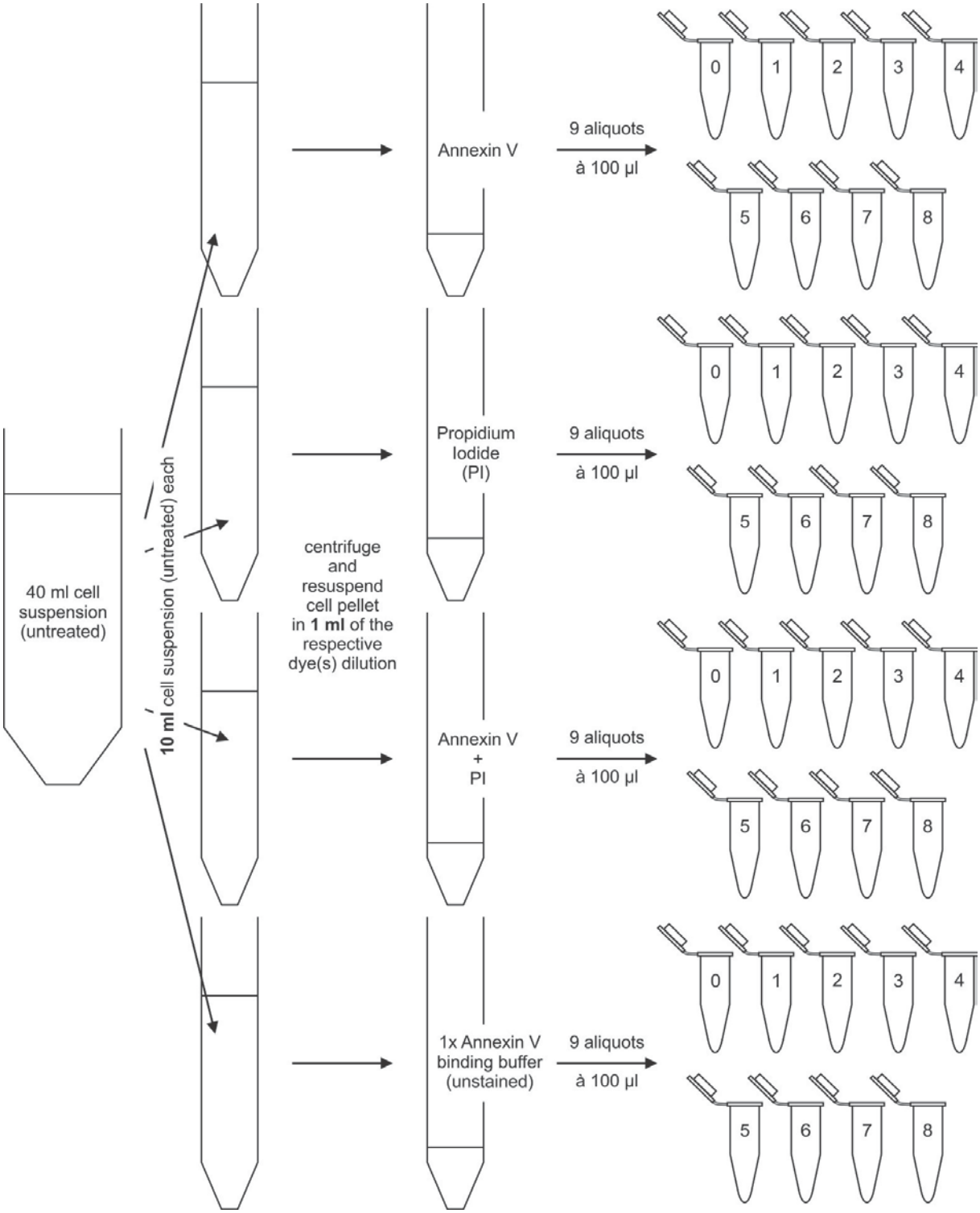


**Figure 4: Cell harvest and staining of untreated samples.** Cells are grown in T75 cell culture flasks, harvested as described above and fused to a homogeneous cell suspension in a 50 ml conical tube. Four aliquots (à 10 ml) of this homogeneous cell suspension are centrifuged and the resulting cell pellets are resuspended in 1 ml of the respective staining solutions (a) Annexin V b) PI c) Annexin V + PI d) 1x Annexin V binding buffer (unstained)). From each of these four differently stained cell suspensions 9 aliquots à 100 µl are made in 1.5 ml microreaction tubes. These receive increasing NM concentrations as described in 6.6.3.2, Table 3 and Figure 5.

- Prepare 9 microreaction tubes (1.5 ml total volume) per staining condition (a-d described above; 36 in total).
- Label these tubes with the respective staining condition and number serially from 0 to 8 (Figure 4 and Figure 5). Numbers refer to the NM (or solvent) concentrations given in Table 3 and the NM dilution series described in 6.5 "Dilution of nanomaterials".

**Table 3: Additives to untreated cells**. A: Concentrations and respective solvent for metal oxide NMs. 10 µl of each NM sub-dilution described in 6.5 "Nanomaterial dilutions" are necessary to reach the final concentrations. B: Concentrations and respective solvent for carbon based NMs. 16 µl of each NM sub-dilution described in 6.5 are necessary to reach the final concentrations. Nothing is added to sample 1. These nine conditions are performed for each of the four staining conditions (a) Annexin V, b) PI, c) Annexin V/PI, d) unstained).

| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| **A** | No | 100 | 50 | 25 | 12.5 | 6.3 | 3.1 | 1.6 | $ddH_2O$ |
| **B** | additive | 80 | 40 | 20 | 10 | 5 | 2.5 | 1.25 | Pluronic |
| | | | | **NM concentrations (µg/ml)** | | | | | **Solvent** |

- Resuspend the four cell pellets in 1 ml of the respective staining solutions (a-c describe above) or 1 ml 1x binding buffer only (unstained).
- Prepare 9 aliquots à 100 µl in the 1.5 ml microreaction tubes.
- Add NM sub-dilutions (as described in 6.5 "Dilution of nanomaterials") as shown in Table 3 and Figure 5.
  A: 10 µl of each sub-dilution of metal oxide NMs are necessary.
  B: 16 µl of each sub-dilution of carbon based NMs are necessary.
  **Note:** All NM dilutions have to be vortexed directly before application to the cells.
- Incubate on ice for 15 min.

**Note:** Time from application of staining solutions to distribution into 9 microreaction tubes to application of NM sub-dilutions should be kept as short as possible!

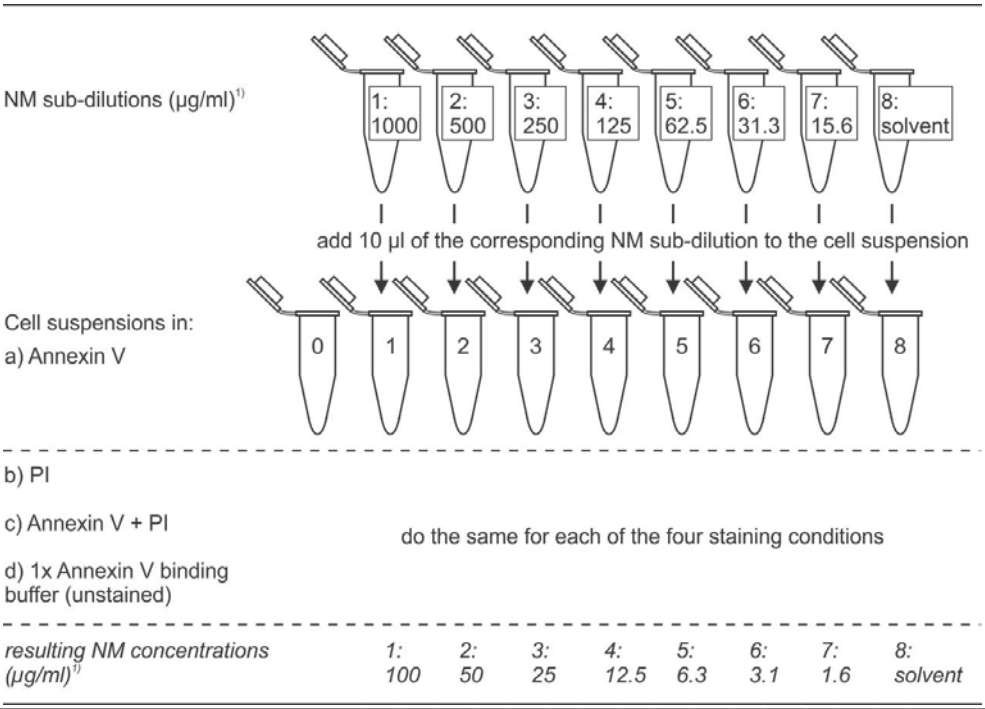


**Figure 5: Addition of NM sub-dilutions.** Preparation of cell suspensions in different staining solutions are described above and illustrated in Figure 4. Here the addition of the respective NM sub-dilutions (as described in 6.5) is shown. 1)NM concentrations given here refer to metal oxide NMs. Carbon based NM concentrations are detailed in the text and in Table 3.

### *6.6.3.3 Preparation of cell free samples*

**Note:** Only the highest concentration of NMs (100 µg/ml for metal oxides, 80 µg/ml for carbon based materials) used in the cellular setup are tested in the cell free controls.

- Label 6 microreaction tubes (1.5 ml) as follows:
  a) Annexin V
  b) PI
  c) Annexin V/PI
  d) 1x Annexin V binding buffer
  e) $ddH_2O$
  f) complete cell culture medium

  a) to c) refers to the staining solutions described in 6.6.1 and Table 2.

- Add 100 µl of the respective staining solution, 1x Annexin V binding buffer, $ddH_2O$ or complete cell culture medium to the tubes.
- Add 10 µl metal oxide stock suspension (1 mg/ml) or 16 µl carbon based NM stock suspension (500 µg/ml) to each of the tubes.
- Mix by vortexing.
- Incubate on ice for 15 min.

## 6.6.4 Flow cytometric analysis

- Record the following parameters in 4 graphs (as shown in Figure 6):
  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.

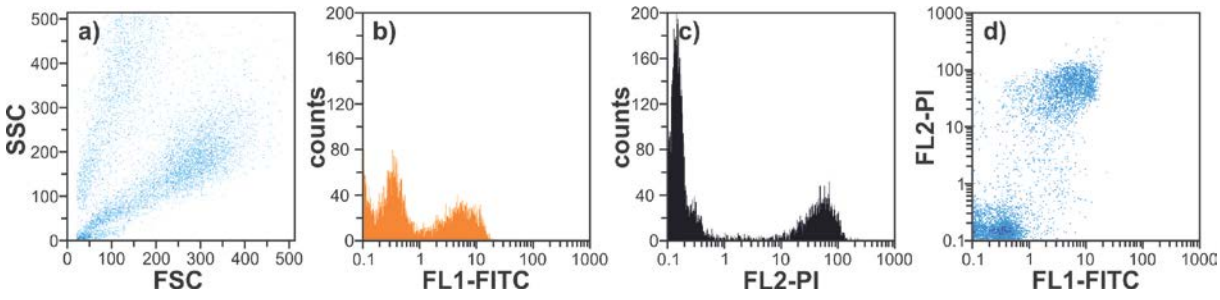


Figure 6: **Data are recorded in four plots.** a) Dot plot recording forward and side scatter to assess cell size and granularity, respectively. b) Histogram FL1-FITC vs. cell counts, detecting the number of Annexin V-FITC positive cells. c) Histogram FL2-PI vs. cell counts, detecting the number of PI 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** (from the 6-well plate) 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 7).
    - 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.

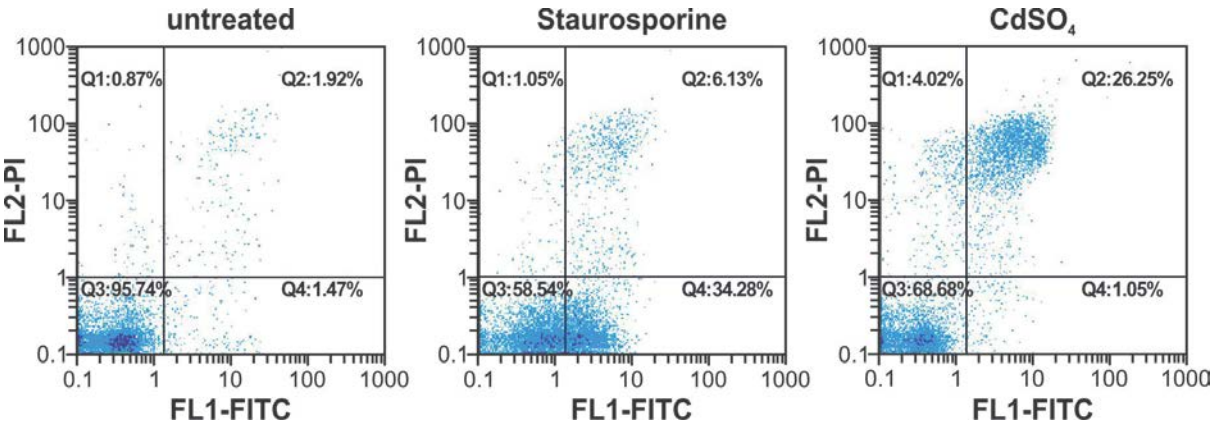


**Figure 7: Example of Annexin V-FITC/PI double labeled A549 cells.** Comparing untreated, Staurosporine and $CdSO_4$ treated AnnexinV/PI double labeled cells illustrates the different modes of cell death and how single and double labeled cells distribute in the dot plot.

- Record Annexin V/PI double labeling data of 10 000 cells per treatment condition (36 microreaction tubes described in 6.6.3.2). 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.

### *6.6.4.1 Recording of cell free controls*

- To record the cell free controls (6.6.3.3) use exactly the same settings as for the cellular controls. Record with the same speed and for the same time!

**Note:** Often no particles can be counted in the cell free controls. To match cell free and cellular conditions as good as possible, recording speed and duration are kept constant.

## 6.7 Data evaluation

Data are presented in a stacked bar chart as shown in Figure 8. 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.

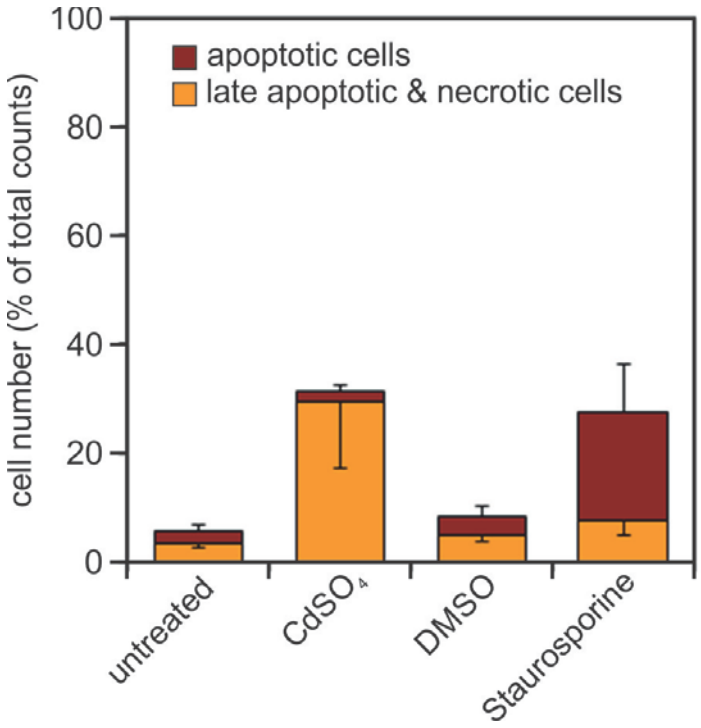


**Figure 8: Example of data presentation in a stacked bar chart.** A549 cells were either left untreated or received 5 mM $CdSO_4$ for 3 h or 4 µM Staurosporine for 4 h. DMSO served as the solvent control for Staurosporine and was also applied for 4 h. Cells were stained with Annexin V-FITC and PI. Shown 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 |
| 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