

# ***MATERIALS AND METHODS***

## ***4 Balb/c3T3 Clone A31-1-1 Cell Line***

The cytotoxicity and morphological transformation studies were carried out using the established and aneuploid mouse fibroblast-like Balb/c3T3 cell line (hereafter named Balb/3T3) from the American Type Culture Collection (ATCC CCL-163), Rockville, MD (USA).

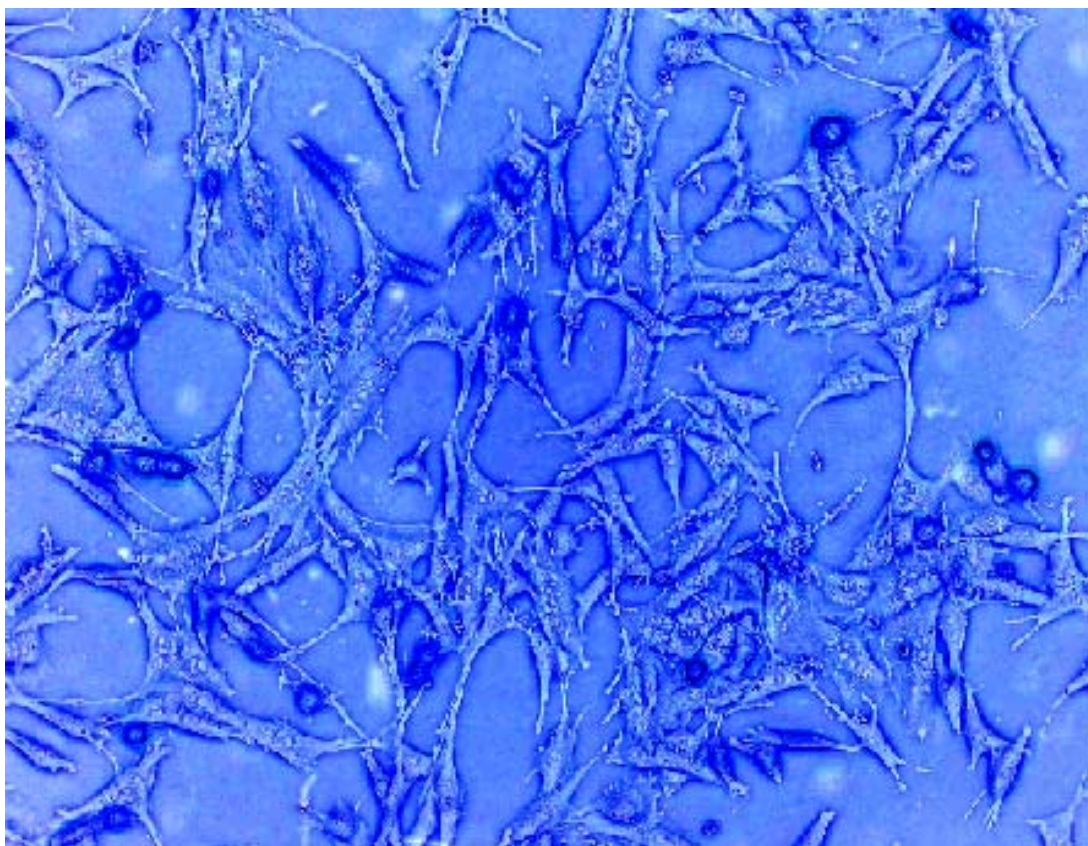
The Balb/3T3 cell line tested was obtained from two different laboratories:

- Laboratory 1 (source 1): ECVAM (passages 8-10).
- Laboratory 2 (source 2): “Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia, Laboratorio Centro Substrati Cellulari” (IZS) of Brescia (Italy) (passages 80-81).

The IZS checked the presence of bacteria and mycoplasma. The cells were contact inhibited and not tumorigenic, and susceptible to transformation by oncogenic DNA SV40 virus and murine sarcoma virus. They were sensitive to herpes simplex, vesicular stomatitis virus (Indiana strain) and Coronavirus, but resistant to type 1 poliovirus and ectromelia virus (mousepox).

### ***4.1 Cell Culture Maintenance***

The first step of the study was the standardisation of the protocol applied to the Balb/3T3 cell line (Photo 4.1), in order to optimise the experimental conditions for cell maintenance and growth on the basis of combinations of reagents and materials.



**Photo 4.1: The Balb/c3T3 A31-1-1 cell line (400X)**

**Growth conditions.** In order to select the best experimental conditions to optimise the cytotoxic response of the Balb/3T3 cells, Colony Forming Efficiency (CFE) experiments were carried out by testing culture media and sera of different origins (Table 4.I).

On the basis of the optimisation of the CFE response, the cells used for testing the metal compounds were grown in 10 ml DMEM with Low-Glucose, sodium piruvate and sodium bicarbonate, without L-glutamine (EUROCLONE, CELBIO), supplemented with 10% FCIII serum (HYCLONE, CELBIO), 4 mM L-glutamine (GIBCO BRL), 2.5 µg/ml amphotericin B (EUROCLONE, CELBIO), 30 U/ml penicillin/30 µg/ml streptomycin (GIBCO BRL), under standardised conditions at 37°C in a 5% CO<sub>2</sub> atmosphere, using a HAEREUS incubator (DASIT, Cornaredo, Milano, Italy).

**Table 4.I: Culture media and sera tested to optimise  
CFE response in Balb/3T3 cells**

<b>Culture medium</b>	<b>Company</b>
Dulbecco's modified Eagle's medium (DMEM) (IX), Low-Glucose, containing L-glutamine, 1000 mg/L D-glucose, sodium piruvate, and 25 mM HEPES	GIBCO BRL (Milano, Italy) Code 22320-022
Dulbecco's modified Eagle's medium (DMEM) (IX), Low-Glucose, containing sodium piruvate and sodium bicarbonate, without L-glutamine (DMEM/LOW)	EURO CLONE Supplied by CELBIO (Pero, Milano, Italy) Code EC M0749L
<b>Serum</b>	<b>Company</b>
Embryonic Stem Cell Qualified FBS (North American Origin)	GIBCO BRL Code 10119-162
Foetal bovine serum (EC approved)	EURO CLONE Supplied by CELBIO Code EC S0180D
Foetal clone III (FCIII, bovine serum product)	HY CLONE Supplied by CELBIO Code SH 30109.02

**Cell culturing.** The Balb/3T3 cells were cultured using: sterile culture dishes 100 x 20 mm (FALCON, Como, Italy); 15 ml and 50 ml sterile tubes (FALCON); 1 ml, 2 ml, 5 ml, 10 ml, 25 ml and 50 ml sterile pipettes (FALCON); pipetman (EPPENDORF, Milano, Italy); P10, P20, P100, P200 and P1000 micropipettes and corresponding tips (EPPENDORF); an aspiration system consisting of a platinum-iridium tube (90% Pt and 10% Ir, 15 cm length, 2 mm diameter, 0.2 mm thickness) (DEGUSSA, Pero, Milano, Italy) that was connected to an aspirator with valve (COSTAR, Concorezzo, Milano, Italy). The aspirator was linked to a sucking/pressing vacuum pump equipped with a neoprene septum (MILLIPORE, Vindrone, Milano, Italy); binocular Olympus CK2 microscope (OLYMPUS, Segrate, Milano, Italy).

The entire study with cells was carried out in sterile conditions, using laminar flow (DASIT) and taking care to work only with sterilised material.

In order not to let stock cultures grow to confluence, the cells were seeded twice a week as described in the following protocol.

When the cells reached 80% of confluence in a 100 x 20 mm culture dish, the culture medium was removed. Then, the cells were rinsed twice with 10 ml PBS Dulbecco's without calcium, magnesium and sodium bicarbonate (GIBCO BRL). Doing so, calcium and magnesium ions were eliminated in order to avoid inhibition of the trypsin action. PBS was replaced with 0.5 ml trypsin-EDTA solution (1X) (SIGMA, Milano, Italy) no more than 2 or 3 minutes until cells are complete detached. A volume of 10 ml culture medium was added to neutralise the trypsin action and the cells were collected in a 15 ml tube and then resuspended. To count the cells, 100  $\mu$ l of cell suspension was prepared using trypan blue solution (0.4%) (SIGMA) and a "Bürker" hemocytometer chamber 0.0025 mm<sup>2</sup>/0.04 mm<sup>2</sup>, 0.100 mm depth (SACCO, Como, Italy). Depending on the cell amount, two different dilution factors were used: df = 2 and df = 10. The cell number/ml was obtained counting all the nine squares of the chamber, without considering the cells touching middle line at bottom and right, and applying the following equation:

$$N = (a / 9) \times df$$

where: N = number of cells/ml;

a = number of cells counted in 9 squares;

df = dilution factor.

The volume of cell solution to seed the required number of cells was calculated by the equation:

$$N : 1 \text{ ml} = b : c$$

where: N = number of cells/ml collected in a tube;

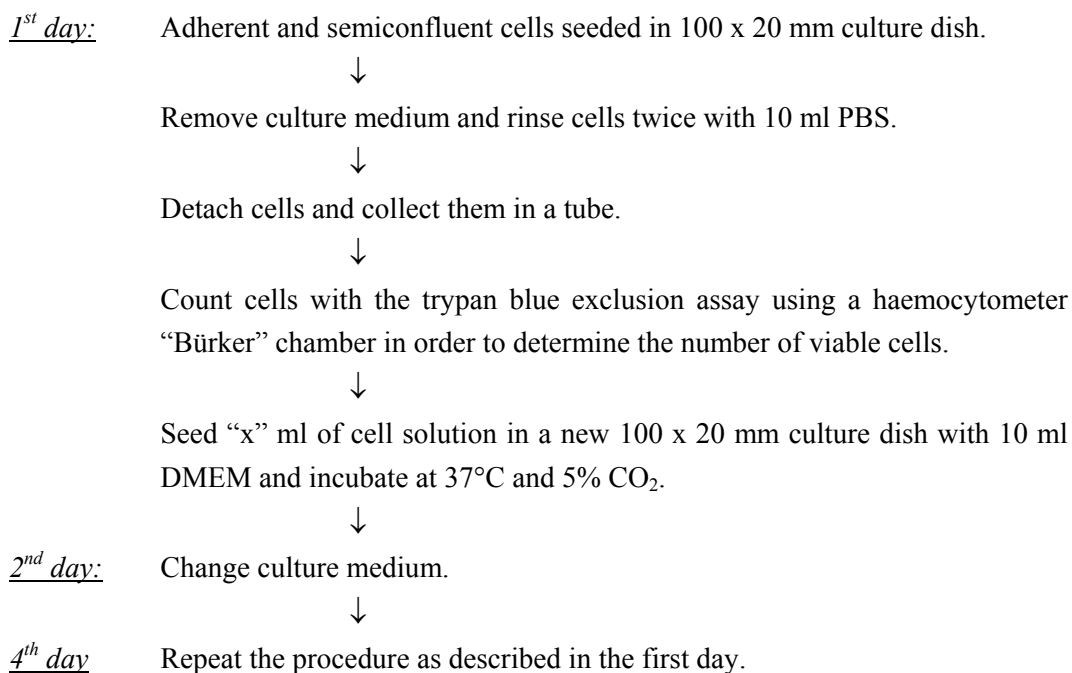
b = number of cells to seed;

c = volume of cell solution to seed.

This volume was seeded in a new 100 x 20 mm culture dish and then 10 ml fresh culture medium was added.

During the entire seeding process, prewarmed washing solutions and culture medium were always used (Figure 4.1).

*Figure 4.1: Seeding method applied to Balb/3T3 cells*



***Cryopreservation of cells.*** Upon counting, cells were rinsed with PBS and centrifuged at 1000 rpm for five minutes. The supernatant was removed and an amount of culture medium was added as the half of the total volume of the plastic vial used for freezing (NUNC - SACCO). Normally, the vial volume varied from 1 ml to 1.8 ml. The other half of the volume consisted of the freezing solution prepared in a 15 ml tube as follows:

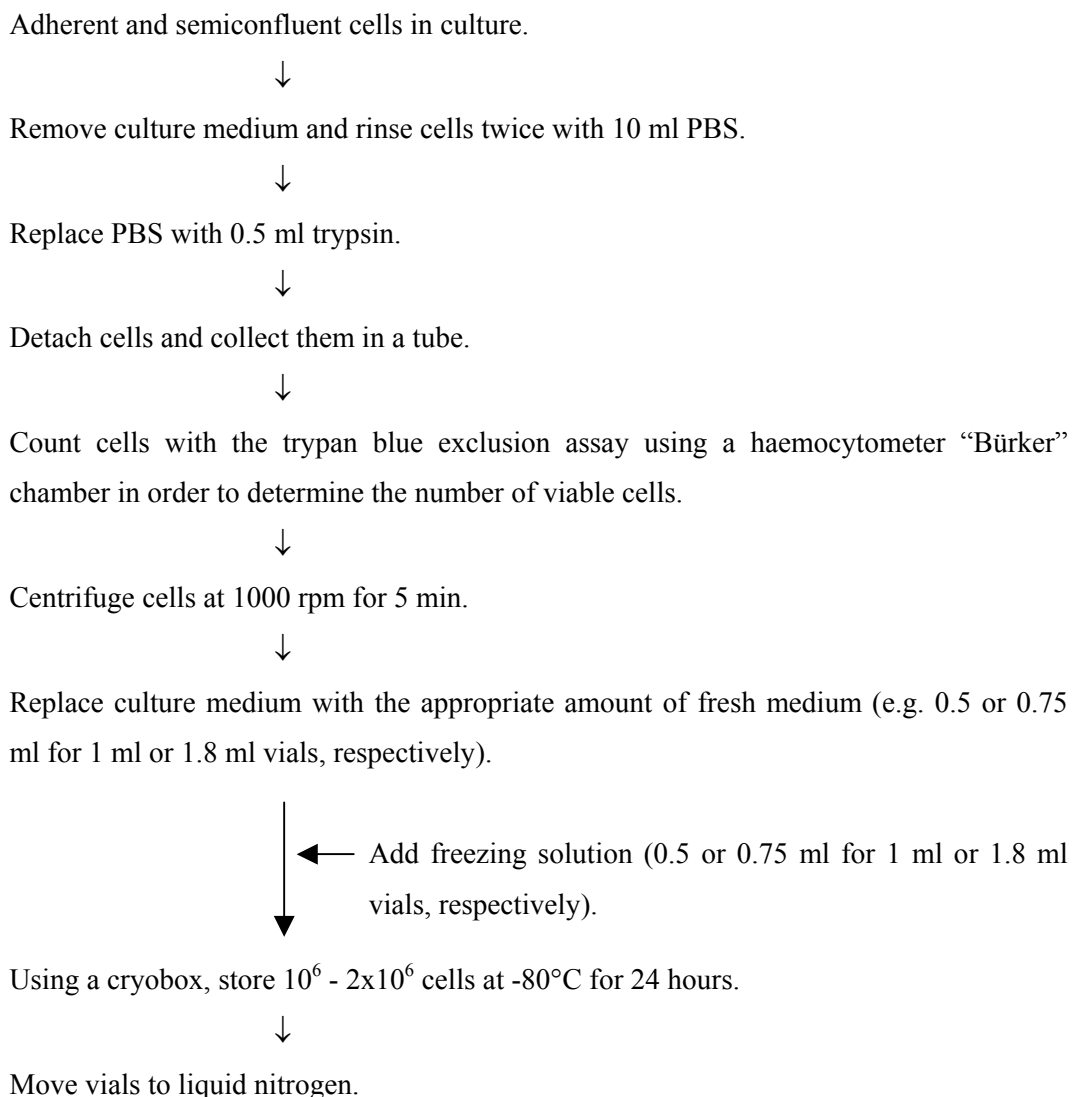
80% culture medium;

10% serum;

10% dimethyl sulfoxide (SIGMA).

In a box with ice every vial was filled firstly with the corresponding volume of freezing solution and then with cell solution. They were put at least for 24 hours in a special cryobox (NALGENE, Milano, Italy) at -80°C and then transferred to liquid nitrogen (Figure 4.2).

**Figure 4.2: Cryomethod applied to Balb/3T3 cells**

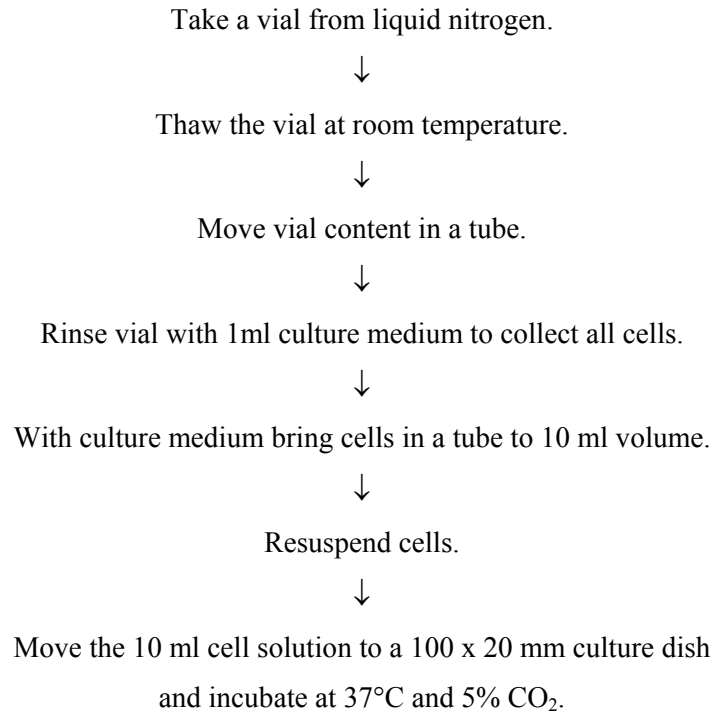


**Cell thawing.** Two different methods for cell thawing were considered. The first one (protocol 1) was a standard method suitable also for other cell lines. The second one (protocol 2) is specific for the Balb/c 3T3 A31-1-1 cell line (Figure 4.3).

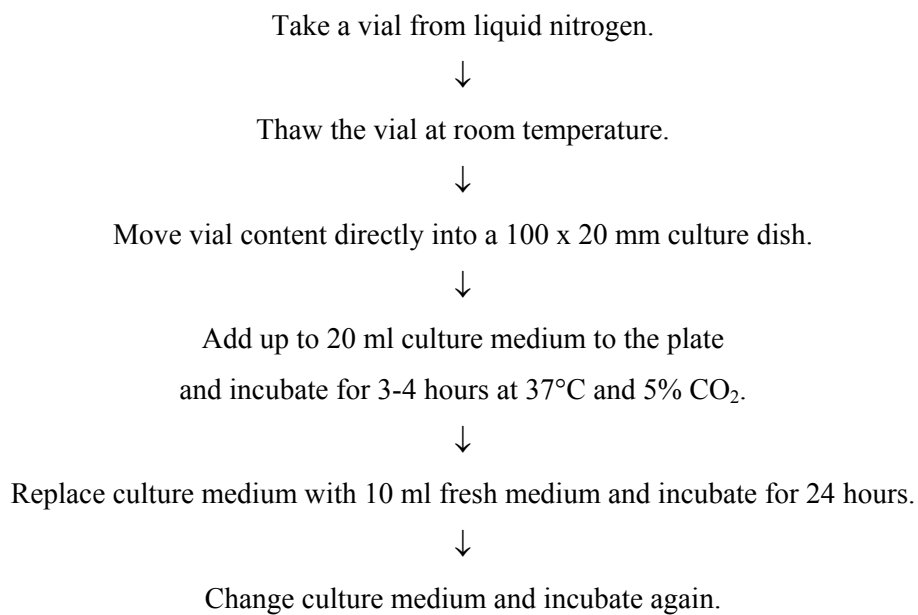
Each method was tested using a frozen vial of Balb/3T3 cells under the same experimental conditions, including the passage number and the amount of cells in each vial. Both cultures were maintained for five days.

**Figure 4.3: Thawing method applied to Balb/3T3 cells**

**a) Standard method:**



**b) Specific method:**





## 4.2 Cytotoxicity: Colony Forming Efficiency (CFE) Assay

According to the adopted protocol (*DiPaolo J.A. et al., 1972*), Balb/3T3 cells were seeded as 200 cells in each 60 x 15 mm culture dish (FALCON) with 4 ml DMEM, using six culture dishes for every treatment. After 24-hour incubation the culture medium was replaced with the metal solution at the specific concentration. After 72-hour exposure the metal solution was replaced with non-treated DMEM that was changed twice every three days. Seven days later the culture medium was removed and the cells were fixed with 4 ml of formaldehyde (37% solution (Formalin; SIGMA)) 10% in PBS for twenty minutes. Then, they were stained with 4 ml of giemsa stain stock solution (0.4%; SIGMA) 10% in H<sub>2</sub>O milliQ for thirty minutes. The plates were rinsed with H<sub>2</sub>O MilliQ and let dry (Figure 4.5).

A stereomicroscope (WILD HEERBRUGG, Switzerland) was used to count colonies with more than 50 cells. The cytotoxicity results were expressed as relative colony forming efficiency (*DiPaolo J.A. et al., 1972*), assuming the number of colonies in treated plates as percent of viability of negative control.

## 4.3 Cytotoxicity: Neutral Red Uptake (NRU) Assay

The neutral red uptake was carried out using the “*In Vitro* Toxicology Assay Kit, Neutral Red Based” kit (SIGMA).

The cells were seeded in 24-well culture dishes (COSTAR).

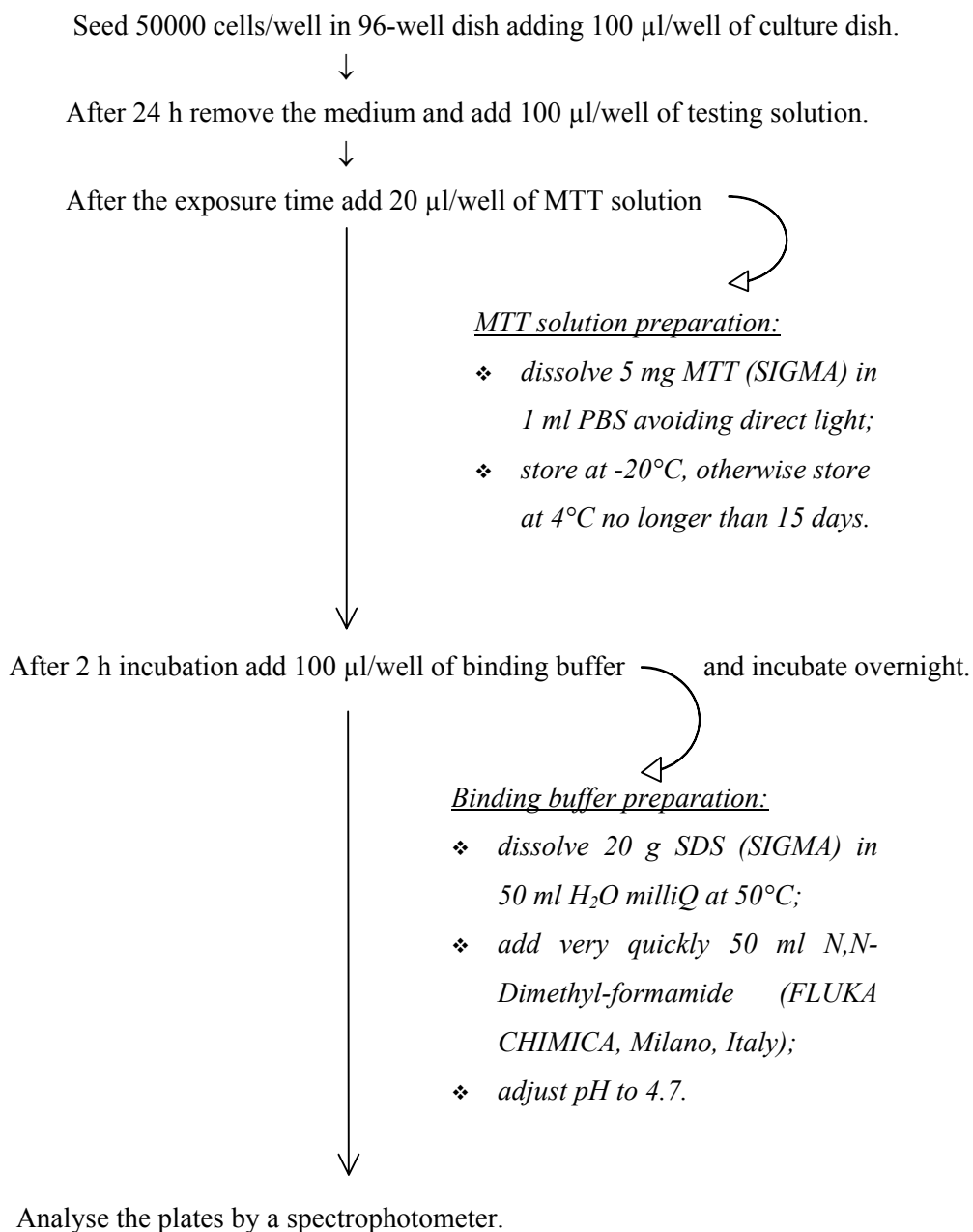
Microcuvettes (Dispolab-KARTELL, Noviglio, Milano, Italy) and a DU-7 spectrophotometer (BECKMAN, Cascina Dé Pecchi, Milano, Italy) were used. The length wave used was  $\lambda = 540$  nm.

#### 4.4 Cytotoxicity: MTT Test

For specific investigations the cytotoxicity of selected metal compounds was studied applying the MTT test (Section 16.1). This colorimetric assay determines the ability of viable cells to convert a soluble tetrazolium salt [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) into an insoluble formazan precipitate. Tetrazolium salts accept electrons from oxidised substrates of appropriate enzymes, such as NADH and NADPH. In particular, MTT is reduced at the ubiquinone and cytochrome b and c sites of the mitochondrial electron transport system and is the result of succinate dehydrogenase. This reaction converts the yellow salts to blue-coloured formazan crystals that can be dissolved in an organic solvent, whose concentration can be determined spectrophotometrically (*Mosmann T., 1983*).

The present test was carried out seeding 50000 cells/well in 96-well culture dishes (FALCON) adding 100 µl/well of culture medium. The wells on the border of the dish were filled up with PBS in order to assure humidity. After 24 h incubation, the culture medium was refreshed with 100 µl/well of testing solution except for the negative control, which was refreshed with non-treated medium. (It is to note that every treatment corresponded to the six wells on the line of the shorter side). At the end of the exposure time, 20 µl MTT solution were added in each well and the culture dishes were incubated for 2 h. To each well, 100 µl binding buffer were added and after overnight incubation optical density at 620 nm was determined in a spectrophotometer Spectra Max 250 (MOLECULAR DEVICES – BIOSPA, Milano, Italy) (Figure 4.4).

**Figure 4.4: Protocol for MTT test and solution preparation**



## 4.5 Concurrent Cytotoxicity and Morphological Transformation Balb/3T3 Assay

The present assay consists of two parts strictly interconnected: cytotoxicity and neoplastic morphological transformation study.

The material used was the same for CFE (Section 4.2).

On the same day the cells were seeded in 4 ml DMEM for each 60 x 15 mm culture dish:

- *cytotoxicity*: 200 cells / culture dish were seeded using six plates for each treatment including negative control (non-treated culture medium) and positive control (known carcinogenic metal compound at specific concentration):
- *morphological transformation*:  $10^4$  cells / culture dish were seeded using 20 plates for each treatment including negative and positive controls.

As previously described in Section 4.2, the CFE was finished after eleven days, while the morphological transformation after five weeks (Figure 4.5). During this period the culture medium was changed twice a week.

The evaluation of the morphological transformation was based on a specific marker, such as the type III foci formation. This kind of analysis was performed with stereoscopic microscopy, taking into account that the features of transformed foci consist of being deep basophilic; having dense multilayering of cells with random cell orientation at any part of the edge; then an invasion into the surrounding contact-inhibited monolayer, and domination of spindle-shaped cells. Cell aggregates that do not meet these criteria were not counted as transformed foci (*IARC/NCI/EPA Working-Group, 1985; Kakunaga T., 1973; Reznikoff C.A. et al., 1973; Rundell J.O., 1984*).

The differences between type I, II and type III foci are related to the degree of morphological aberrations associated with each type. Recommended criteria for scoring these foci are detailed in Table 4.II (*IARC/NCI/EPA Working-Group, 1985*), from which it is possible to conclude that type III foci more than 2 mm in diameter have three phenotypic properties: a) piling and overlapping cells; b) disorientation of cells at the periphery of the focus; and c) invasion of transformed cells into a contact-inhibited monolayer of wild-type cells. Type I and type II foci of Balb/3T3 cells are also recorded. They appear in many different sizes, but they lack the combination of three phenotypic properties previously noted for the type III transformed focus (*Matthews*

*E.J., 1993c*). Probably these properties make able type III foci to induce neoplastic formation in nude mice with a frequency of 85% (*Saffiotti U. et al., 1984*) (Photos 4.2-4.4).

Thus, with regard to the evaluation of the transforming potential of metal compounds, the present work consider only the type III foci that are the basis to establish the following parameters:

- the ratio between the total number of type III foci per treatment and the number of plates of the same treatment;
- the total number of type III foci scored in all the plates of the treatment;
- the ratio between the number of the positive plates and the total number of the plates in that treatment.

From the analytical point of view the transforming potential (transformation frequency, Tf) was calculated by dividing the number of type III foci scored in each treatment with the number of cells surviving for the same treatment.

**Table 4.II: Features of type I, II and III foci**

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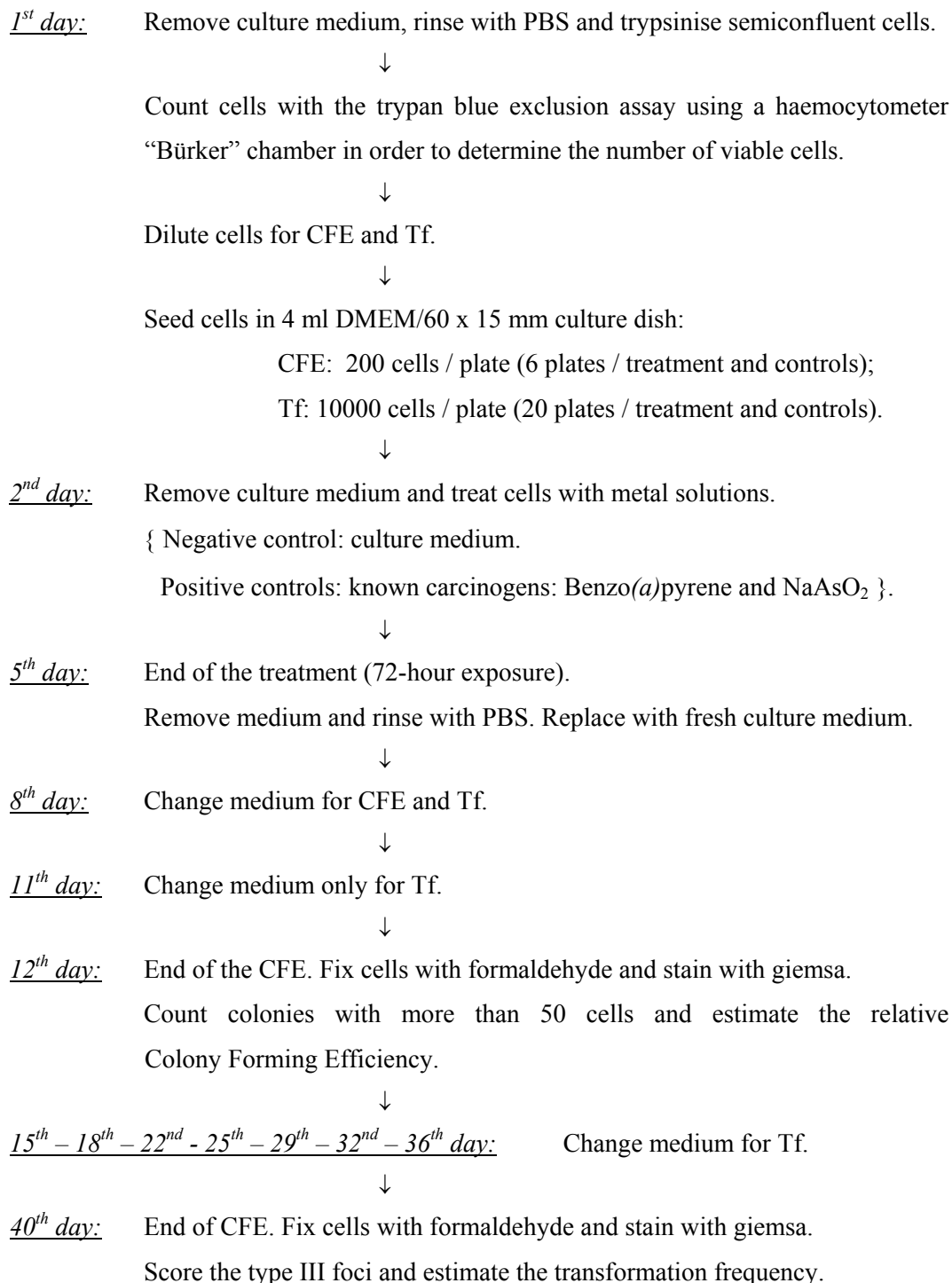
<ul style="list-style-type: none"> <li>◆ <i>Type III foci have the following properties:</i> <ul style="list-style-type: none"> <li>▪ <i>they appear dense, multilayered and basophilic;</i></li> <li>▪ <i>they show random orientation at focus edge, and invasion into the monolayer;</i></li> <li>▪ <i>the transformed cells are predominantly spindle-shaped.</i></li> </ul> </li> <li>◆ <i>Type II foci are distinguishable from type III foci primarily because of the more ordered and defined edge. They are dense, multilayered, and less basophilic than type III. These differences are much more accentuated in type I foci, which show a very low overlapping and piling up of cells.</i></li> <li>◆ <i>Usually “mixed” foci having both type II and type III morphologies should be scored as type III. Mixed foci that have both type I and type II properties should be scored as type II.</i></li> <li>◆ <i>Note should be taken of unusual transformed foci (e.g. corded, banded, poorly attached) especially if they represent a substantial fraction of the foci, and caution should be exercised in including these in the total number of transformed foci.</i></li> <li>◆ <i>Focus size depends on culture conditions and assay duration. Generally, foci of less than 1 or 2 mm should not be scored. However, small foci with striking type III transformed morphology could be counted as transformed at the discretion of the investigator.</i></li> </ul>
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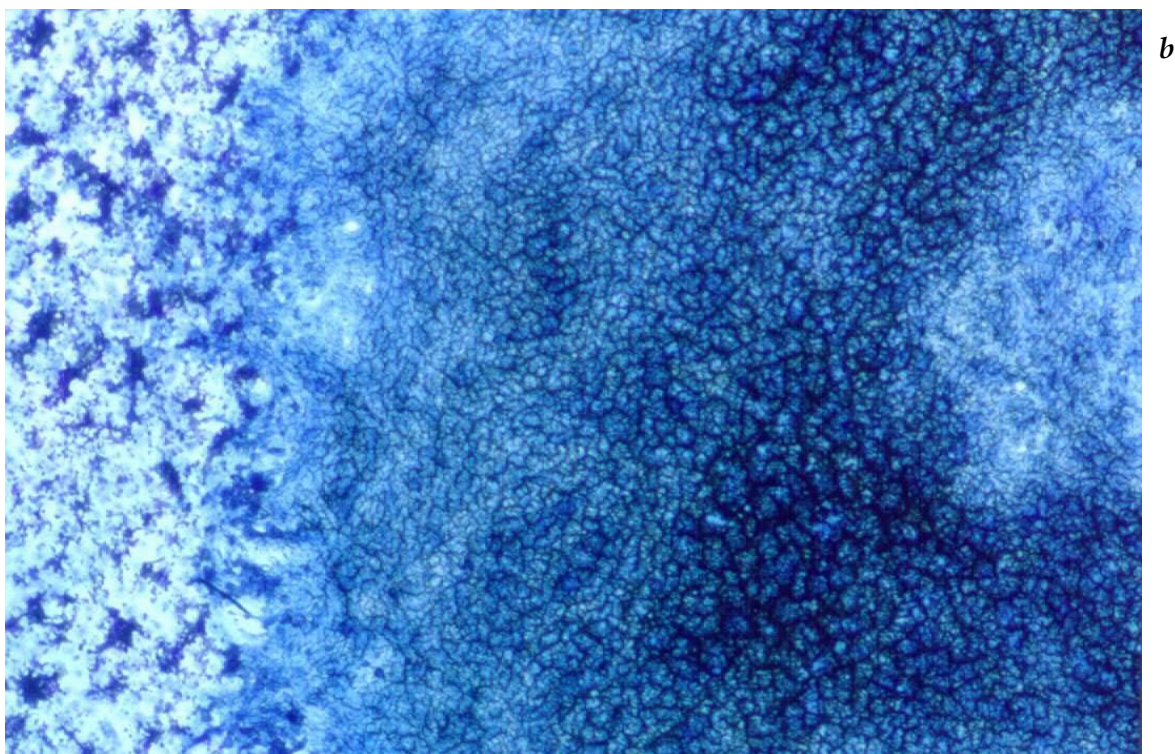
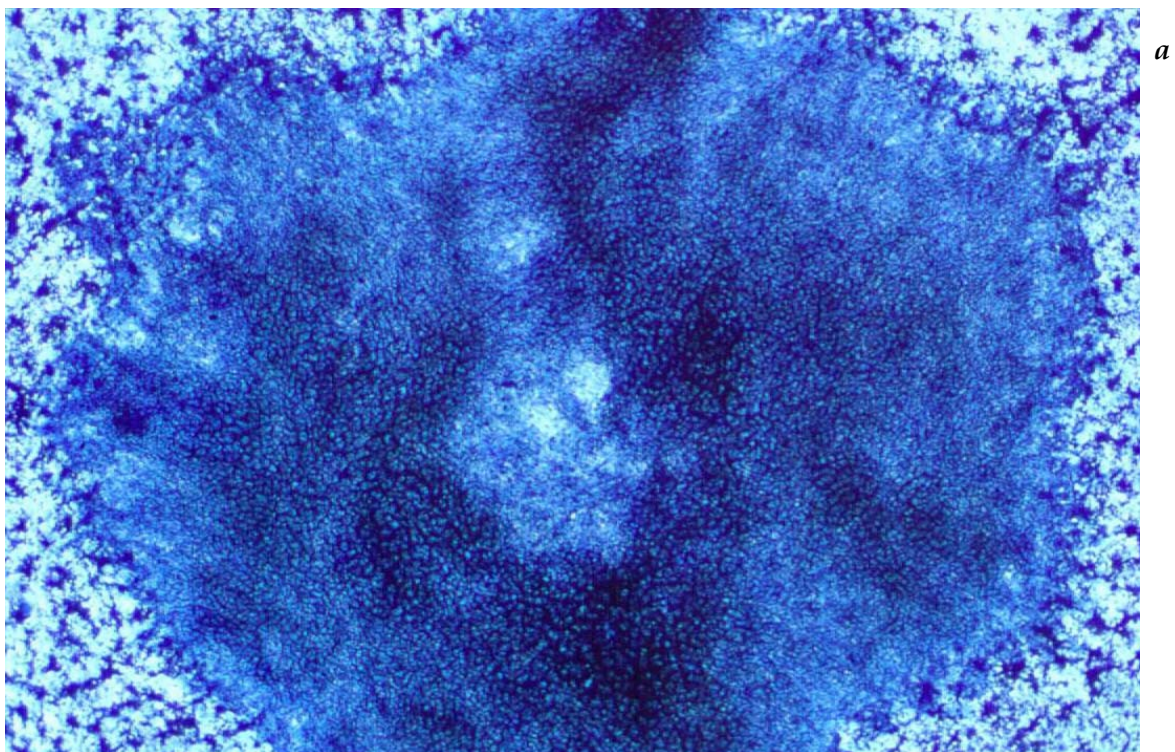


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**Figure 4.5: Protocol of concurrent cytotoxicity (CFE)  
and morphological transformation Balb/3T3 assay**

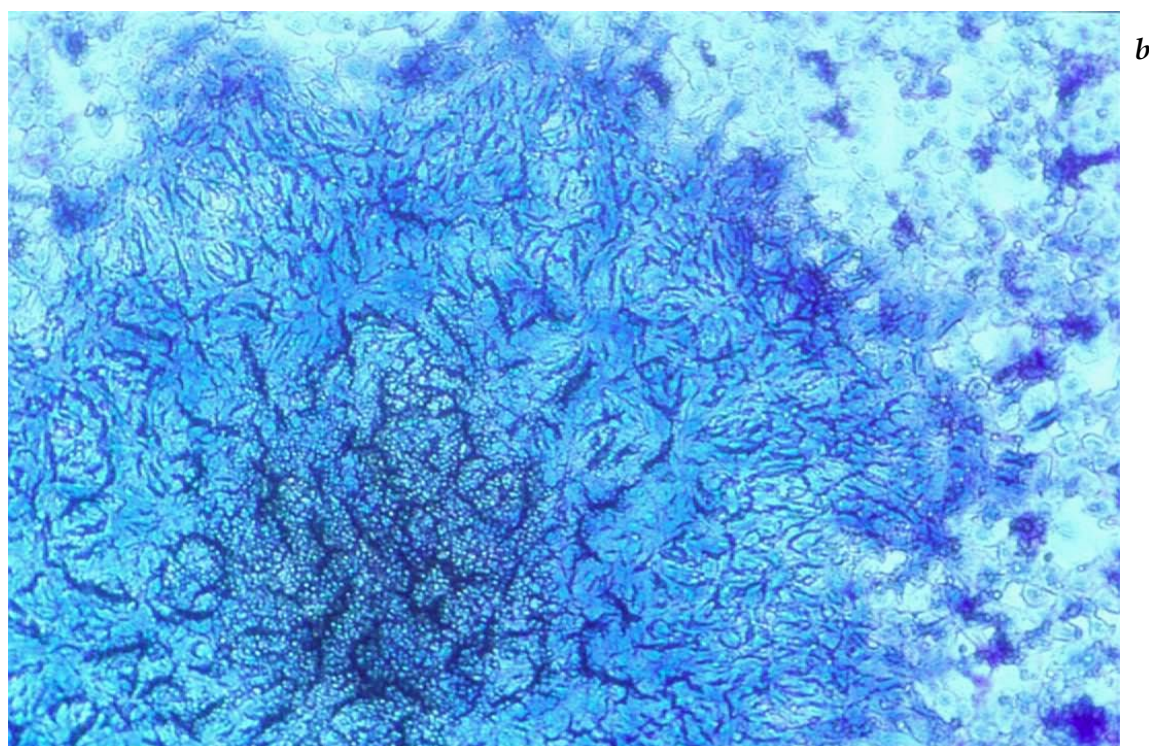
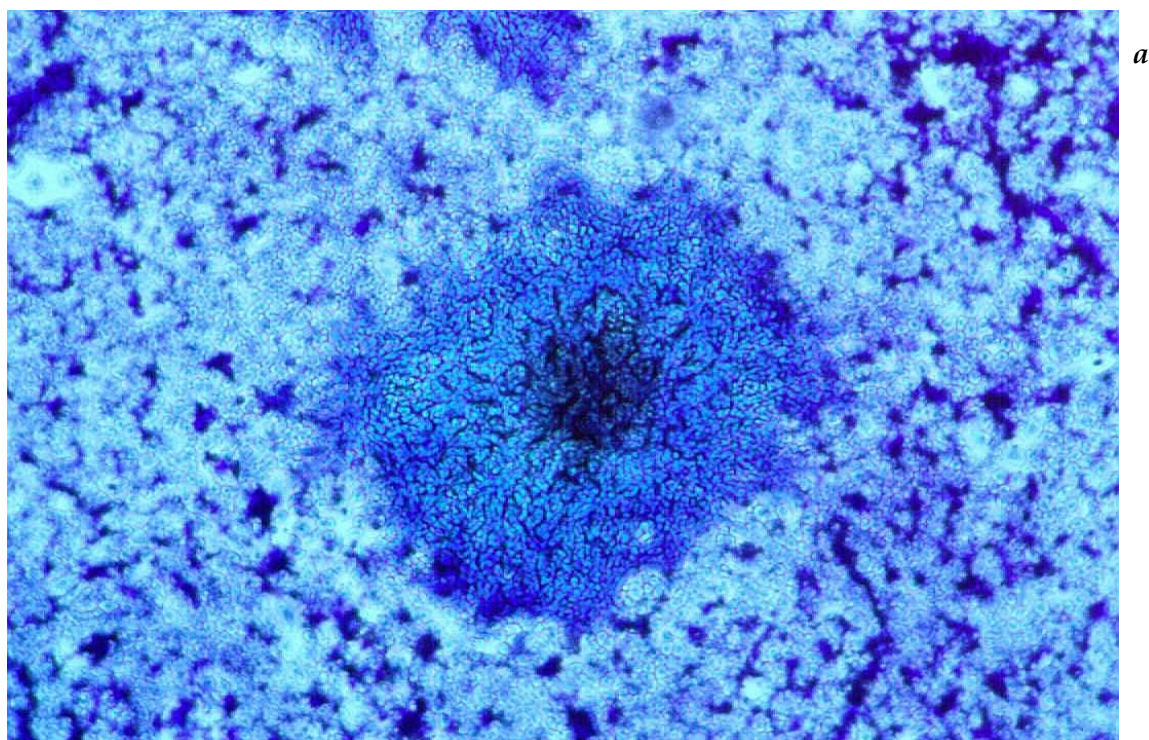


**Note.** If the CFE is performed independently, the protocol is the same until the 12<sup>th</sup> day.



*Photo 4.2: a) type I focus induced in BALB/3T3 cells exposed to 5  $\mu$ M  $(\text{NH}_4)_2\text{PtCl}_6$  (100X);*

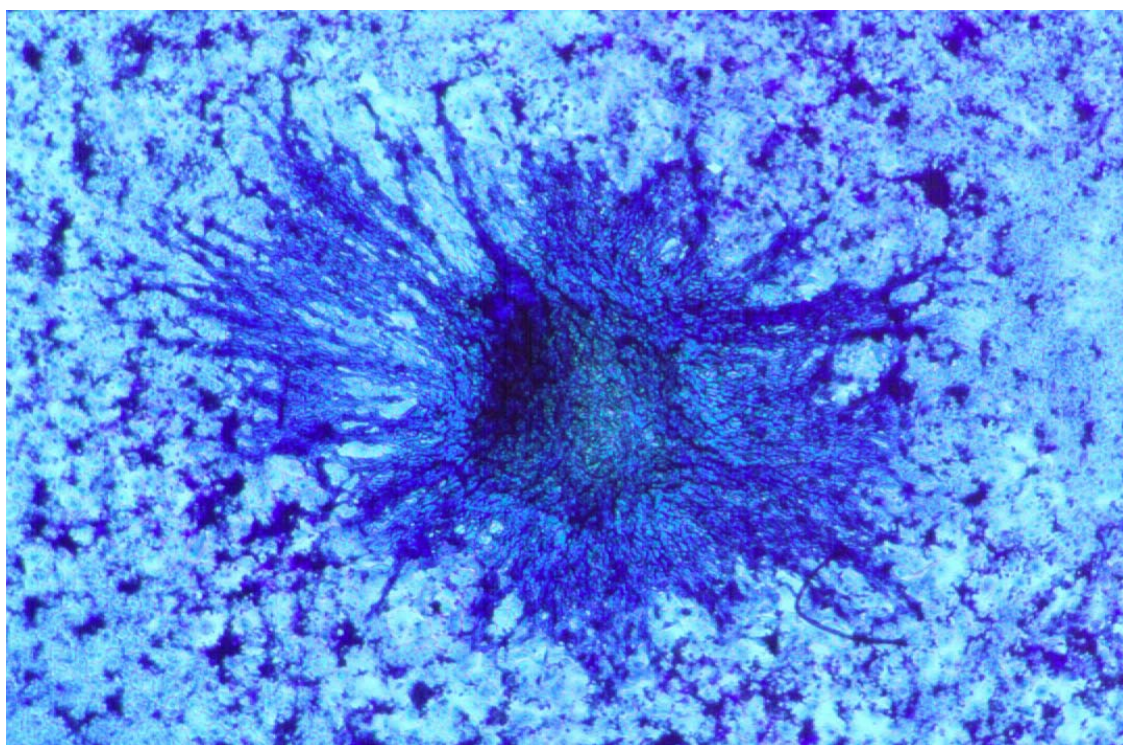
*b) a detail (200X)*



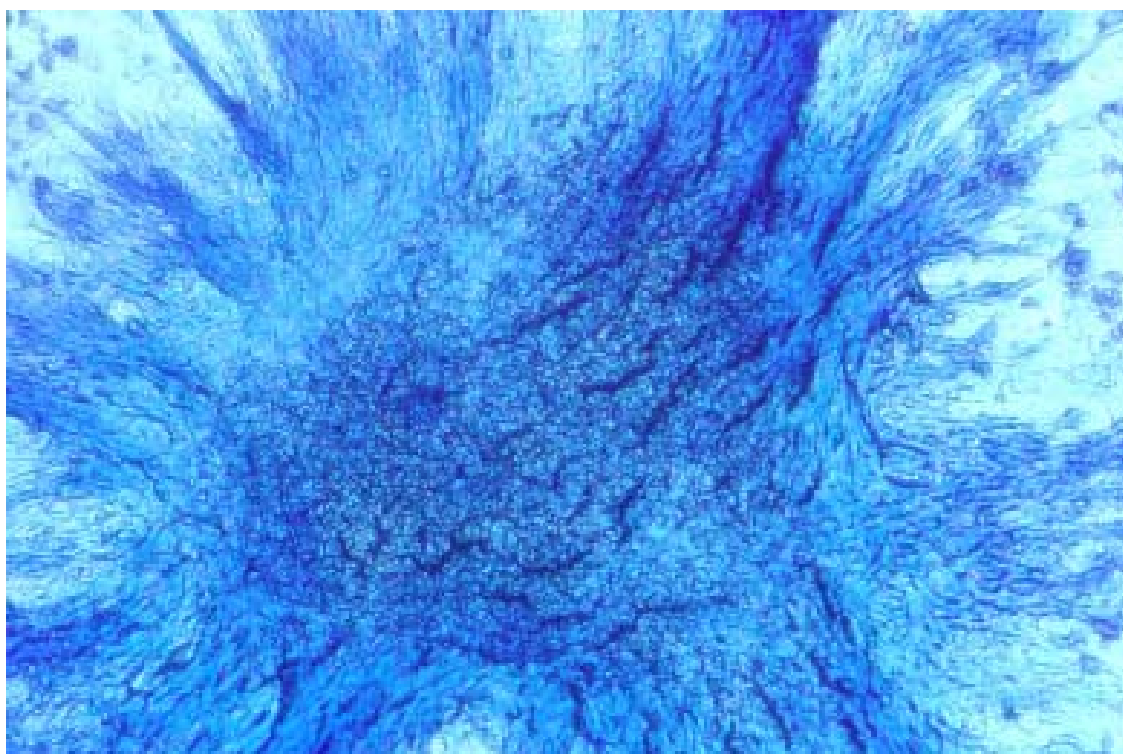
*Photo 4.3: a) type II focus induced in BALB/3T3 cells exposed to 5  $\mu$ M  $(\text{NH}_4)_2\text{PtCl}_6$  (200X);*

*b) a detail (400X)*





*a*



*b*

*Photo 4.4: a) type III transformed focus induced in Balb/3T3 cells exposed to 5  $\mu\text{M}$   $(\text{NH}_4)_2\text{PtCl}_6$  (200X)*

*b) a detail of type III transformed focus induced in Balb/3T3 cells exposed to 7  $\mu\text{M}$   $(\text{NH}_4)_2\text{PtCl}_6$  (400X)*

*Note the random orientation at focus edge, the predominant spindle-shape and the invasion into the monolayer*

## 4.6 Cloning

At the end of the morphological transformation experiments, some type III foci were cloned in order to obtain individual metal-transformed cell lines.

The adopted protocol (*Reid L.C.M., 1979*) is described in Figure 4.6.

**Figure 4.6: Protocol for cloning anchorage-dependent cells applied to the Balb/3T3 cell line**

Remove culture medium from plates and rinse them twice with PBS. Leave a residue of PBS over the cells to prevent their drying.



Sterilise forceps dipping into 70% ethanol; dip previously sterilised cloning rings into the culture dish containing stopcock grease (alternatively, cloning rings with grease-coated ends (SIGMA) were used). Place the grease-coated end on the culture dish bottom to let it seal with the plastic just around a cell colony (type III focus).



Add trypsin to each ring: usually 1-2 drops are enough to fill the ring.



Incubate each plate containing cloning rings for 5 min. at 37°C and periodically observe them using a phase microscope. When the cells of the type III focus are rounded and ready to be detached, use a P100 pipette and pipette the solution gently up and down until complete detachment of cells.



Pipette the solution of suspended cells into a 24-well culture dish (FALCON). Add 2 ml of cloning medium per well.



Single cell suspensions were then prepared from monolayer cultures as follows:

- Remove culture medium from the 24-well culture dishes and rinse the wells twice with PBS;
- add 200-300 µl of trypsin to each well and incubate culture dishes at 37°C for 5 min. It should be better to minimise the trypsination time;
- as soon as the majority of cells are rounded and mostly detached from the plates, squirt the cells with culture medium.



Count cells with the trypan blue exclusion assay in order to determine the number of viable cells.



Dilute cell suspension to a concentration of 10 cells/ml.



Distribute 0.1 ml of the medium containing cells into each well of a 96-well culture dish (FALCON). Ensure homogeneous dispersion of the cells throughout the medium.



Carefully observe each well, then score and chose those wells containing only one cell. Incubate the cultures.



During cell growth it should be unnecessary to change the medium. However, if colony formation appears to be slow the spent medium can be gently aspirated from cultures and refreshed with new one.



Cultures deriving from only one cell were expanded until 75 cm<sup>2</sup> flasks (COSTAR) and then frozen as stocks of 10<sup>6</sup> - 2x10<sup>6</sup> cells.

#### 4.7 Cell Cultures in Soft Agar

To assess the tumorigenic activity of the Balb/3T3 clones in agar medium, two different concentrations, 1% and 0.6% agar noble (SIGMA), were used and alternatively mixed with Modified Eagle Medium (2X) liquid with L-glutamine and without Phenol Red (GIBCO) supplemented with 20% FCIII serum (HYCLONE, CELBIO) and 30 U/ml penicillin / 30 µg/ml streptomycin (GIBCO). The protocol applied is described in Figure 4.7.

**Figure 4.7: Protocol for soft agar plating method**

Prepare 1% and 0.6% agar solution.



Thaw the 1% agar in a microwave and stabilise it at 45°C for 15-30 min.



Mix 9 ml of 1% agar with 9 ml complete MEM and put 3 ml of this mixture in each well of a 6-wells culture dish (FALCON). During this procedure take care to avoid air bubbles formation. Let dry this layer.



Count  $10^6$  cells from the initial culture and suspend them in 2.25 ml MEM.



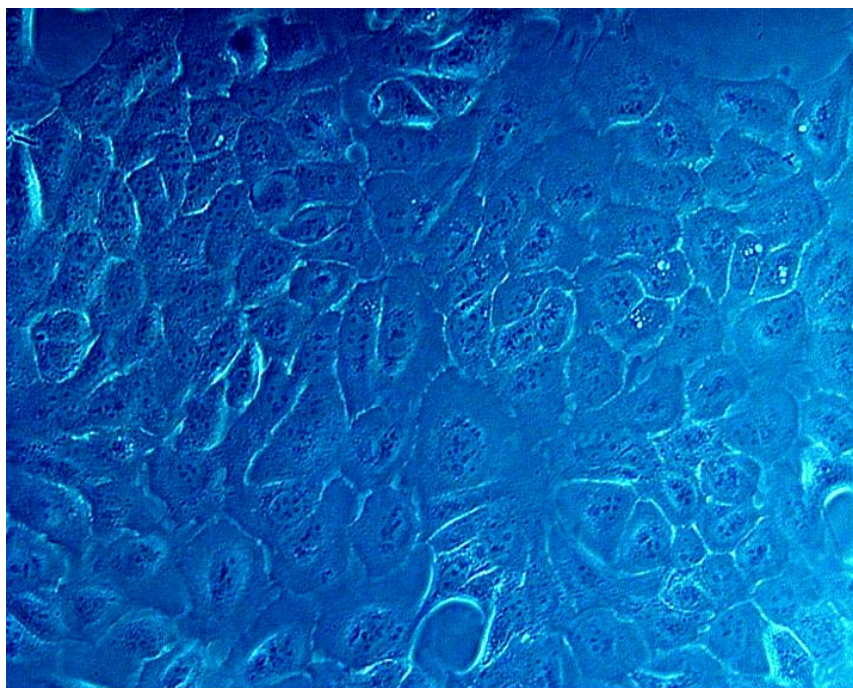
Thaw and then stabilise the 0.6% agar.



Mix 2.25 ml of the cell solution with 2.25 ml of 0.6% agar and drop 0.75 ml of the mixture over the solidified 1% agar. Let dry this second layer before incubating.

## 5 *HaCaT Cell Line*

The HaCaT cell line was supplied by Prof. Norbert E. Fusenig from DKFZ (Deutsches Krebsforschungszentrum - Im Neuenheimer Feld, Heidelberg, Deutschland) (Photo 5.1). These cells were grown in DMEM with High-Glucose, sodium pyruvate and L-glutamine (EUROCLONE, CELBIO), supplemented with 10% FCII serum (HYCLONE, CELBIO), 2 mM L-glutamine (GIBCO BRL), 30 U/ml penicillin/30 µg/ml streptomycin (GIBCO BRL), under standardised conditions at 37°C in a 5% CO<sub>2</sub> atmosphere, using a HAEREUS incubator (DASIT). The culturing procedure related to cell maintenance and CFE experiments were performed under sterile conditions (Sections 4.1 and 4.2). Unlike the Balb/3T3 cell line, for the HaCaT cells much longer time was necessary to detach the cells with trypsin. Finally, cells were thawed on the basis of the chosen standard protocol adopted for the Balb/3T3 cells (Figure 4.3a).



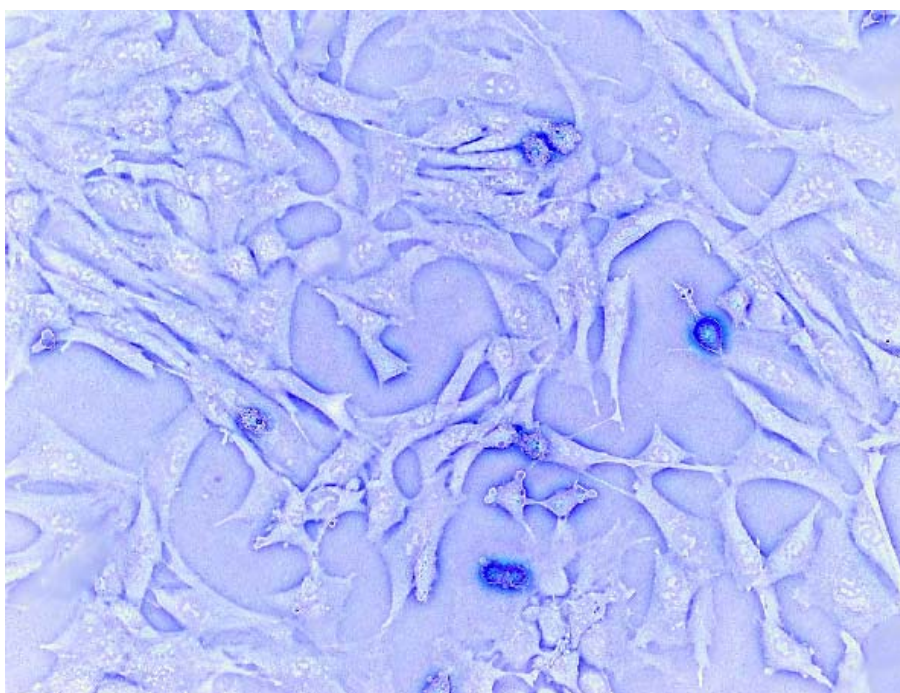
*Photo 5.1: The HaCaT cell line (400X)*

### 5.1 *Cytotoxicity: Colony Forming Efficiency (CFE) Assay*

The adopted method was the same applied on the Balb/3T3 cell line as described previously in Section 4.2.

## 6 Syrian Hamster Embryo Cell Line

Both feeder and target Syrian Hamster Embryo (SHE) cells were purchased from “Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia, Laboratorio Centro Substrati Cellulari” (IZS) of Brescia (Italy), at the passages 2-3. They were guaranteed as free of bacteria and fungi (Photo 6.1).



*Photo 6.1: The Syrian Hamster Embryo cell line (400X)*

### 6.1 Cell Cultures Maintenance

**Growth conditions.** In order to reproduce the recommendations of the LeBoeuf’s protocol (Kerckaert G.A. *et al.*, 1996), the present study on the SHE cells was mostly focused on the optimisation of the experimental conditions adopted in the CFE assay. For this reason the experiments were carried out without testing any metal solution.

However, for a better understanding of this *in vitro* rodent transformation assay, the entire protocol is described in Section 6.2.

Table 6.I reports culture medium and sera tested.

*Table 6.I: Culture medium and sera tested for the SHE cell line*

<b>Culture medium</b>	<b>Manufacturer</b>
Minimum essential medium, with Earle's salts and 2g/l sodium piruvate (MEM/EBSS)	EURO CLONE Supplied by CELBIO  Code EC B2071L
<b>Serum</b>	<b>Manufacturer</b>
Foetal bovine serum (Canada FDA approved) Lot. STF 654	EURO CLONE Supplied by CELBIO  Code EC S0150L
Foetal bovine serum (EC approved) (origin depends on batch numbers)	EURO CLONE Supplied by CELBIO  Code EC S0180L

The SHE cells were grown in MEM with Earle's Salts and 2g/l sodium piruvate (EUROCLONE, CELBIO), supplemented with 20% FBS - Canada FDA approved (EUROCLONE, CELBIO), 4 mM L-glutamine (GIBCO BRL), 30 U/ml penicillin/30 µg/ml streptomycin (GIBCO BRL), 5 ml non-essential amino acids solution 100X (EUROCLONE, CELBIO), under standardised conditions at 37°C in a 10% CO<sub>2</sub> atmosphere, using a HAEREUS incubator (DASIT).

**Culture maintenance.** IZS sacrificed pregnant (13 day gestation) Syrian golden hamsters. Usually 3-4 animals were killed to obtain 40-50 embryos for the cell isolation procedure.

For the SHE assay, two types of cells, target and feeder cells, are distinguished. Target cells remain metabolically and reproductively active throughout the assay, and

can be morphologically transformed by treatment. However, they have a very low cloning efficiency (i.e. approx. 1-3%). To improve this characteristic, the target cells are seeded onto a layer of inactivated (X-irradiated) feeder cells, which can no longer divide but can provide factors to support the growth of the target cells and improve cloning efficiency to 20% or more (*Dunkel V.C. et al., 1991*). IZS commissioned the exposure of normal SHE cells to X-ray irradiation (~5000 rad) in order to obtain the feeder cells. Both feeder and target cells were supplied in frozen vials.

All the procedures related to the routine maintenance, such as trypsinisation, rinsing, counting, as well as thawing and freezing of cells, items and plastic materials were the same as those used for the Balb/3T3 cell line (Section 4.1).

Despite the suggestions reported in the LeBoeuf's protocol (*Kerckaert G.A. et al., 1996*), every treatment with the SHE cells was performed at the original pH value of culture medium: pH = 7.4.

## **6.2 Cytotoxicity and Morphological Transformation Assays**

Even though in the present work neither the cytotoxicity nor the morphological transformation assays were carried out to test metal compounds, details on their performance are provided as follows.

Before performing the SHE cell transformation assay, a cytotoxicity assay is carried out to establish an appropriate concentration-range for the testing metal compounds. The concentration for transformation studies is selected on the basis of IC<sub>50</sub> values.

The same concentration protocol selected for the transformation assay (24-hour or 7-day exposure – see below) should be used for the cytotoxicity assay. This involves exposure of the SHE cells to the test chemical in clonal growth for a time period (24 hours or 7 days) to a range of concentrations of test chemical.

For every treatment the number of plates to be used is: a) 15 culture dishes / concentration of test chemical plus 15 dishes both for negative and positive control groups, normally including 24-hour and 7-day test chemical exposure for the CFE assay; b) 20 culture dishes / concentration of test chemical plus 20 dishes both for



negative and positive control groups. In every case (*a* and *b*) five dishes containing only feeder cells were included in each assay to verify the inability of these cells to replicate.

The protocol for the cytotoxicity and the morphological transformation assays is described in Figure 6.1.

**Figure 6.1: Protocol for cytotoxicity and morphological transformation assays applied on SHE cells**

1<sup>st</sup> day:     **Seeding of X-irradiated feeder cells.**

Remove culture medium, then rinse with PBS and trypsinise semiconfluent cells.

↓

Count cells with the trypan blue exclusion assay using a haemocytometer “Bürker” chamber in order to determine the number of viable cells.

↓

Dilute cells to obtain  $4 \times 10^4$  -  $6 \times 10^4$  cells / 2 ml

↓

Seed the 2 ml cell suspension in 60 x 15 mm culture dish:

CFE: 15 plates / treatment and control plus 5 plates only with feeder cells both for 24-hour and for 7-day exposure.

Tf: 20 plates / treatment and control plus 5 plates only with feeder cells both for 24-hour and for 7-day exposure.

↓

Incubate feeder cells for 24 hours.

↓

2<sup>nd</sup> day:     **Seeding of target cells.**

Remove culture medium, then rinse with PBS and trypsinise semiconfluent cells.

↓

Count cells with the trypan blue exclusion assay using a haemocytometer “Bürker” chamber in order to determine the number of viable cells.

↓

Dilute cells to obtain 300 cells / 2 ml

↓

Seed the 2 ml cell suspension in 60 x 15 mm culture dish containing the X-irradiated feeder cells and incubate target cells for 24 hours.

↓

3<sup>rd</sup> day: Treat cells adding 4 ml metal solution to obtain 8 ml of total medium/dish.

{ Negative control: culture medium.

Positive controls: known carcinogens: Benzo(a)pyrene and NaAsO<sub>2</sub> }.

↓

4<sup>th</sup> day: End of the 24-hour exposure.

Remove medium and rinse with PBS. Replace with 8 ml fresh culture medium. Let the 7-day exposure undisturbed.

↓

10<sup>th</sup> day: End of the 7-day exposure.

Fix both exposure times (24 hours and 7 days) with formaldehyde and stain with giemsa.

Count the colonies with more than 50 cells and estimate:

- Mean of colonies / culture dish.
- Total number of colonies / test group.
- Mean plating efficiency (PE) ± SEM, where  $PE = (\text{number colonies/dish} \div \text{number of cells seeded}) \times 100$ .
- Relative plating efficiency (RPE) where  $RPE = (\text{test group PE} \div \text{solvent control PE}) \times 100$ .
- Number of morphologically transformed (MT) colonies.
- MT frequency =  $(\text{number of MT colonies} \div \text{total number of colonies}) \times 100$ .
- Mean number of cells / dish / test group.
- Colony density =  $\text{mean number of cells / dish} \div \text{mean number of colonies / dish}$ .
- Relative colony density =  $(\text{colony density test group} \div \text{colony density of solvent control}) \times 100$ .

## 7 Metal Compounds and Solutions

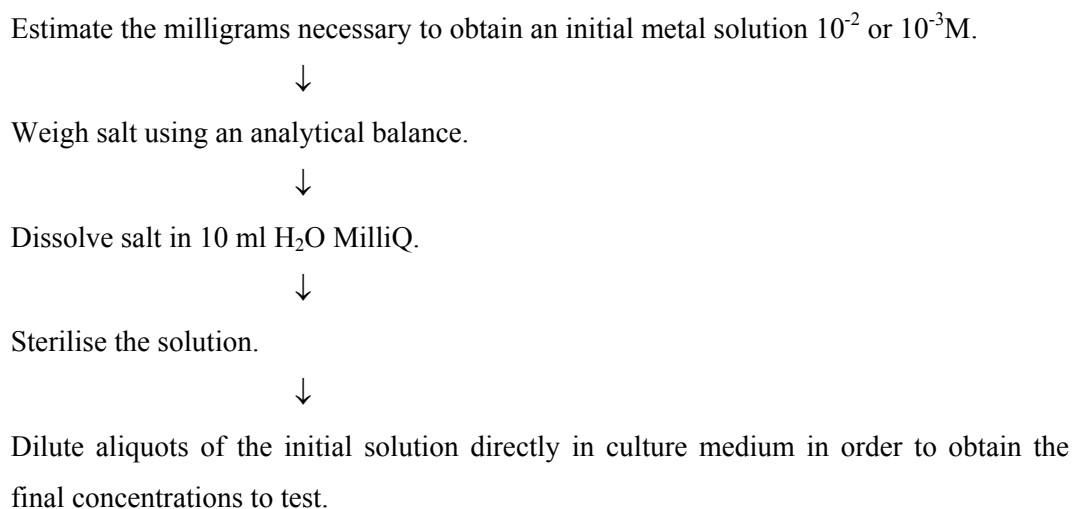
Metal species tested for cytotoxicity and carcinogenicity studies were the following:  $\text{AgNO}_3$ ,  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ,  $\text{KAsF}_6$ ,  $\text{LiAsF}_6$ ,  $\text{NaAsF}_6$ ,  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ ,  $(\text{CH}_3)_3\text{AsCH}_2\text{COO}^-$  (As-betaine),  $\text{AuCl}_3$ ,  $\text{Ba}(\text{NO}_3)_2$ ,  $\text{BeCl}_2$ ,  $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ ,  $\text{H}_3\text{BrO}_3$ ,  $\text{KBr}$ ,  $\text{KBrO}_3$ ,  $\text{CdCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CdMoO}_4$ ,  $\text{Ce}(\text{NO}_3)_3 \cdot \text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ,  $\text{CsCl}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{LiF}$ ,  $\text{HfCl}_2 \cdot \text{O} \cdot 8\text{H}_2\text{O}$ ,  $\text{HgCl}_2$ ,  $\text{Ga}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{GeO}_2$ ,  $\text{NaI}$ ,  $\text{InCl}_3 \cdot 2\text{-}3\text{H}_2\text{O}$ ,  $(\text{NH}_4)_3\text{IrCl}_6 \cdot \text{H}_2\text{O}$ ,  $(\text{NH}_4)_2\text{IrCl}_6$ ,  $\text{La}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{K}_2\text{MoO}_4$ ,  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ ,  $\text{NbCl}_5$ ,  $\text{NiCl}_2$ ,  $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $(\text{NH}_4)_2\text{OsCl}_6$ ,  $\text{Pb}(\text{NO}_3)_2$ ,  $(\text{NH}_4)_2\text{PdCl}_4$ ,  $(\text{NH}_4)_2\text{PdCl}_6$ ,  $\text{Na}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$ ,  $\text{Na}_2\text{PtBr}_6 \cdot 6\text{H}_2\text{O}$ ,  $\text{Na}_2\text{Pt}(\text{OH})_6$ ,  $\text{Na}_2\text{PtI}_6 \cdot 6\text{H}_2\text{O}$ ,  $(\text{NH}_4)_2\text{PtCl}_4$ ,  $(\text{NH}_4)_2\text{PtCl}_6$ ,  $\text{PtCl}_2$ ,  $\text{PtCl}_4$ , *cis*- $(\text{NH}_3)_2\text{PtCl}_2$  (*cis*-diamminedichloroplatinum(II), *cis*-Pt),  $\text{RbCl}$ ,  $\text{NH}_4\text{ReO}_4$ ,  $(\text{NH}_4)_3\text{RhCl}_6$ ,  $(\text{NH}_4)_2[\text{Ru}(\text{H}_2\text{O})\text{Cl}_5]$ ,  $\text{K}_4\text{Sb}_2\text{O}_7$ ,  $\text{Na}_2\text{SeO}_3$ ,  $\text{Na}_2\text{SeO}_4$ ,  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{Sr}(\text{NO}_3)_2$ ,  $\text{Na}_2\text{TeO}_4 \cdot 2\text{H}_2\text{O}$ ,  $\text{Na}_2\text{TeO}_3$ ,  $\text{K}_2\text{TeO}_3 \cdot \text{H}_2\text{O}$ ,  $\text{K}_2\text{TiO}_3$ ,  $(\text{C}_5\text{H}_5)_2\text{TiCl}_2$  (titanocene),  $(\text{NH}_4)_2[\text{TiO}(\text{C}_2\text{O}_4)_2] \cdot \text{H}_2\text{O}$  (titanium oxalate),  $\text{Th}(\text{NO}_3)_4 \cdot 8\text{H}_2\text{O}$ ,  $(\text{C}_5\text{H}_5)_2\text{VCl}_2$  (vanadocene),  $\text{NaVO}_3 \cdot \text{H}_2\text{O}$ ,  $\text{K}_2\text{WO}_4$ ,  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{Zr}(\text{NO}_3)_4$  were purchased from ALFACHEM, (Cologno Monzese, Milano, Italy);  $\text{NaAsO}_2$ ,  $(\text{C}_6\text{H}_5)_4\text{AsCl} \cdot \text{H}_2\text{O}$  (tetraphenylarsonium chloride hydrate),  $(\text{C}_6\text{H}_5)_3\text{AsO}$  (triphenylarsine oxide),  $(\text{CH}_3)_2\text{AsNaO}_2 \cdot 3\text{H}_2\text{O}$  (dimethylarsinic acid sodium salt, DMA),  $\text{Na}_2\text{CrO}_4 \cdot 4\text{H}_2\text{O}$ ,  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  and  $\text{VOSO}_4 \cdot 5\text{H}_2\text{O}$ , from FLUKA CHIMICA; Benzo(*a*)pyrene,  $\text{CH}_3\text{HgCl}$  (monomethylmercury) and *cis*- $\text{C}_4\text{H}_6(\text{COO})_2\text{Pt}(\text{NH}_3)_2$  (*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II), carbo-Pt) from SIGMA-ALDRICH (Milano, Italy);  $(\text{CH}_3)_3\text{AsO}$  (trimethylarsine oxide),  $(\text{CH}_3)_4\text{AsI}$  (trimethylarsonium iodide),  $((\text{CH}_3)_3\text{AsCH}_2\text{CH}_2\text{OH})\text{Br}$  (As-choline) and  $\text{CH}_3\text{AsO}(\text{OH})_2$  (monomethylarsonic acid, MMA) from TRI CHEMICAL LABORATORY (Osaka, Japan).

Whenever possible, metal salts were dissolved in MilliQ water (sometimes at 30°C or more frequently using an electrical vortex) at the concentration of  $10^{-2}$  M or  $10^{-3}$  M, weighing the compounds with an analytical balance AG 245 METTLER TOLEDO (Greifensee, Switzerland). Every metal solution was filtered under laminar flow using a 10 ml sterile syringe (SACCO) and a 0.22  $\mu\text{m}$  filter (MILLIPORE).

Aliquots of the concentrated metal solutions (usually stored at +4°C) were added to complete culture medium in order to obtain the final concentrations to test in the

experiments. After 24 hours, Balb/3T3 cells were incubated with the prewarmed metal-containing medium (Figure 7.1).

**Figure 7.1: Protocol for preparation of metal solutions**



## 7.1 Elemental Analysis of Impurities

The ICP-MS technique was used to test eventual salt impurity in some metal solutions of particular interest in this work.

The inductively coupled plasma-mass spectrometry (ICP-MS) is a multi-elementar technique successfully applied to trace metals when studied in biological samples. This technique is based on the combination of two technologies in series: a) an available inductively coupled plasma (8000-10000 K) to generate ions; and b) a mass spectrometry to evaluate the generated ions. Working in “omni range”, this analytical instrument enables concentration assessment of eight order of magnitude. The range of values is from fraction of ppt (ng/l) to ten of ppb ( $\mu\text{g/l}$ ).

The sequence of events is as follows. The sample is sucked by a peristaltic pump, nebulised in a “cross flow” box and then quickly vaporised in the central part of the

plasma. Through the plasma the dissociation is quite complete because the ionisation potential of most of the elements is less than 10 eV and in these conditions the ionisation is quite complete (>90%). The produced ions enter in a high vacuum box ( $\sim 10^{-5}$  mmHg) by means of two cones (named “sampler” and “skimmer”) used as interfaces of the box with the plasma. Finally the ions are led to a mass detector, sequentially filtered by a “photostoper” and the bars form the quadrupole.

The ICP-MS determines metal concentrations as ultratraces with an order of magnitude of ng/l. Moreover, this technique has good reproducibility and efficiently assures a considerable precision of estimations (RSD < 3%) (Durrant S.F., 1992; Pietra R. et al., 1994). The ICP-MS analysis of Pt-compounds has been carried out as previously described (Farina M., 2003) using an ICP-MS Sciex/Perkin-Elmer Elan 6000 (PERKIN ELMER Life Sciences, Milano, Italy).

## 7.2 Solubility Studies

The palladium solubility of  $(\text{NH}_4)_2\text{PdCl}_4$  and  $(\text{NH}_4)_2\text{PdCl}_6$  (oxidation states +2 and +4, respectively) was checked by dissolving each salt in culture medium with or without serum. After 72-hour incubation the four samples were ultracentrifuged (40000 x g/h) and the Pd-content of the supernatant was analysed by ICP-MS (Section 7.1).

# 8 Metabolic Studies

## 8.1 Preparation of Radiochemicals and Radiolabelling

$^{73}\text{As}$ -arsenic carrier-free ( $T_{1/2} = 17$  days) was supplied by LOS ALAMOS National Lab (NM, USA) as arsenic(V) acid in 0.1M HCl.  $^{73}\text{As(III)}$ - and  $^{73}\text{As(V)}$ -labelled solutions were prepared by adding the  $^{73}\text{As}$  radiotracer in trivalent or pentavalent oxidation state to aliquots of stable  $\text{NaAsO}_2$  or  $\text{Na}_2\text{HAsO}_4$  solutions at the final concentration requested. The oxidation states of the resulting solutions were tested

by paper electrophoresis ( $> 99.5\%$  in both cases) before their use (Sabbioni E. *et al.*, 1987).

$^{51}\text{Cr}$  radiotracer ( $T_{1/2} = 28$  days) was supplied by PERKIN ELMER Life Sciences in aqueous solution as sodium chromate. The specific radioactivity was  $180 \mu\text{Ci}$  of  $^{51}\text{Cr}/\mu\text{g}$  of Cr. Before its use the chemical form was checked by the method of Minoia (Minoia C. *et al.*, 1983). More than  $99.9\%$  of Cr was in the hexavalent form  $\text{Na}_2[^{51}\text{Cr}]\text{CrO}_4$ .

$^{73}\text{As}$  and  $^{51}\text{Cr}$  radioactivity were counted by integral- $\gamma$  counting (Wizard 3, PERKIN ELMER Life Sciences). Each time radioactivity measurements of  $^{73}\text{As}$  and  $^{51}\text{Cr}$  were interpreted in terms of exogenous metal concentration by comparing them with that of reference solutions of  $^{73}\text{As}$  and  $^{51}\text{Cr}$  with known specific radioactivity.

## 8.2 Pt-uptake

Experiments were carried out by exposing Balb/3T3 cells for 72 hours to different concentrations from  $0.1 \mu\text{M}$  to  $10 \mu\text{M}$  of  $(\text{NH}_4)_2\text{PtCl}_6$ ,  $\text{PtCl}_4$ ,  $\text{PtCl}_2$ , carbo-Pt and *cis*-Pt, in order to assess the Pt-content. After exposure and rinsing three times with PBS buffer, the cells were mineralised with  $\text{HNO}_3$  in teflon boxes. The molarity of the acid in the final solution was adjusted to 2 M and then this solution was injected in the ICP-MS (Section 7.1) for Pt determination.

## 8.3 As-uptake and Intracellular Repartition

Subconfluent Balb/3T3 cells, growing in  $75 \text{ cm}^2$  flasks, were exposed in 4 ml of complete culture medium (DMEM) to different concentrations of inorganic  $^{73}\text{As}(\text{III})$  or  $^{73}\text{As}(\text{V})$  ranging from  $0.6 \mu\text{M}$  to  $30 \mu\text{M}$ . After different exposure times, the medium was removed and the adherent cells were washed three times with 20 ml PBS and detached with a trypsin-EDTA solution ( $0.25$  and  $0.02\%$  w/v respectively). In the uptake studies four flasks were used for each characterisation. Viable cells were counted

and the metal uptake in intact cells was determined by measurement of  $^{73}\text{As}$  incorporated into the cells by integral- $\gamma$  counting.

The intracellular repartition of the cellular metals between organelles (pellet fraction) and cytosol (supernatant fraction) was determined after disruption of cells by sonication and centrifugation at 105000 x g for 90 minutes in a refrigerated miniultracentrifuge TL 100 (BECKMANN, Milano, Italy) to separate the organelle-free cytosol. Arsenic incorporated into pellets as well as in cytosols was determined by the approach above mentioned for the determination of total As in the intact cells. The experiments were repeated at least three times.

#### **8.4 Biotransformation of Cr in Growth Medium**

DMEM culture medium was incubated with 1  $\mu\text{M}$  of  $\text{Na}_2\text{CrO}_4$  plus 0.1  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  in the same chemical form. After 72 h, 0.5 ml HCl was added to 1 ml of medium and incubated with 1 ml of liquid resin LA1 Amberlite and 1 ml methylisobutylketon. The mixture was stirred by centrifugation at 3000 rpm for 2 minutes.  $^{51}\text{Cr}$  was measured in both phases, the  $^{51}\text{Cr(VI)}$  being present in the organic phase (*Minoia C. et al., 1983*).

## **9 Studies on Apoptosis**

### **9.1 Annexin V/PI Assay**

Plasma membrane phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane. In live cells phosphatidylserine (PS) is almost exclusively observed on the inner surface of the membrane. Loss of phospholipid asymmetry leading to exposure of PS on the outside of the plasma membrane is an early event in apoptosis. Annexin V, a  $\text{Ca}^{2+}$ -dependent phospholipid-binding protein, has high affinity for negatively charged phospholipids like PS. This protein, when conjugated with a fluorochrome, can be used as a sensitive probe for the

presence of PS on the outside of plasma membrane. Translocation of PS to the external cell surface is not unique to apoptosis, but occurs also during cell necrosis. However, by using a combination of annexin V-FITC conjugate and propidium iodide (PI), it is possible to distinguish apoptotic from necrotic cells with flow cytometry. During apoptosis, the cells become reactive with annexin V after the onset of chromatin condensation but prior to the loss of the plasma membrane's ability to exclude cationic dyes, such as PI. Thus, non-apoptotic cells are annexin V- and PI-negative (FITC<sup>-</sup>/PI<sup>-</sup>), early apoptotic cells are annexin V-positive but PI-negative (FITC<sup>+</sup>/PI<sup>-</sup>), and late apoptotic cells as well as necrotic cells are stained intensively with PI (*Koopman G. et al., 1994; Vermes I. et al., 1995*) (Figure 9.1).

In the present study, 200000 cells/well were seeded in 6-well culture dish (FALCON) adding 3 ml/well of culture medium. After 24 h from cell seeding, the medium was replaced by 3 ml/well of testing solution. Only the negative control was refreshed with non-treated medium. At selected concentrations each metal compound was tested after 2-, 6-, 12-, and 24-hour exposure.

Samples were prepared in accordance with the recommendations provided from the Annexin V-FITC Kit (code n° PN IM3546, IMMUNOTECH, Merseille, France):

at the end of the exposure time attached and suspended cells were collected and testing solutions washed out. Cell pellet was resuspended in the supplied binding buffer and then this solution was incubated with the annexin V-FITC and the PI solutions. Afterwards, each cell sample was analysed by flow cytometry (Epics Elite ESP, COULTER, Milano, Italy).

For a better performance of the assay, during cell collection the exposure to trypsin solution (purposely diluted to 0.28 g/l) was reduced to a minimum, while the centrifugation speed was lower than usual (750 rpm compared with 1000 rpm). Then, the PI concentration was decreased from 250 µg/ml to 125 µg/ml.

In the analysis by flow cytometry, fluorescence compensation was carried out prior to setting up the cytometric protocol for FITC and PI. FITC was collected in PMT2 (green) and PI in PMT4 (red) upon excitation at 488 nm. The conditions for



fluorescence compensation are summarised as follows:

- Tubes were prepared with single stained cells (for both probes), unstained cells and double stained cells.
- The compensation tube consists of cells that are unstained as well as cells that are singly stained with the fluorescent probes.
- The best compensation was attained by mixing, in roughly equal proportions, unstained cells with an aliquot of the same cells stained with the appropriate probe(s).
- A positive control was used through out.
- The compensation was adjusted until the median (log) of the positives is equal to the median of the negatives. This was carried out individually for each stain, and then controlled for the double (FITC and PI) stained cells.

Cells were gated on Side Scatter (SS) versus Forward Scatter (FS), so as to exclude debris, and distinguished as follows (Figure 9.1a):

living cells - (A - FITC<sup>-</sup>PI<sup>-</sup>);

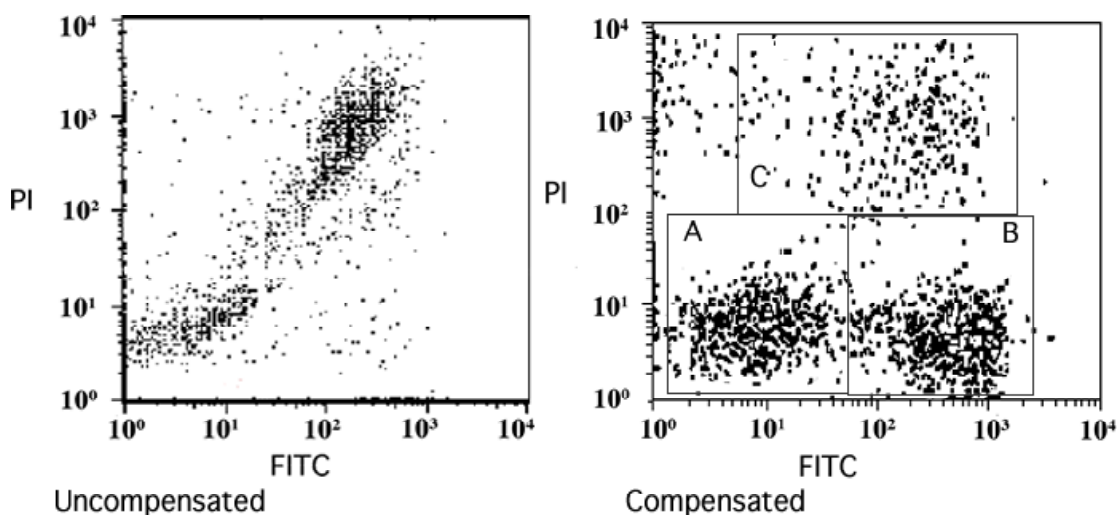
early apoptotic (B - FITC<sup>+</sup>PI<sup>-</sup>);

late apoptotic or necrotic cells (C - FITC<sup>+</sup>PI<sup>+</sup>).

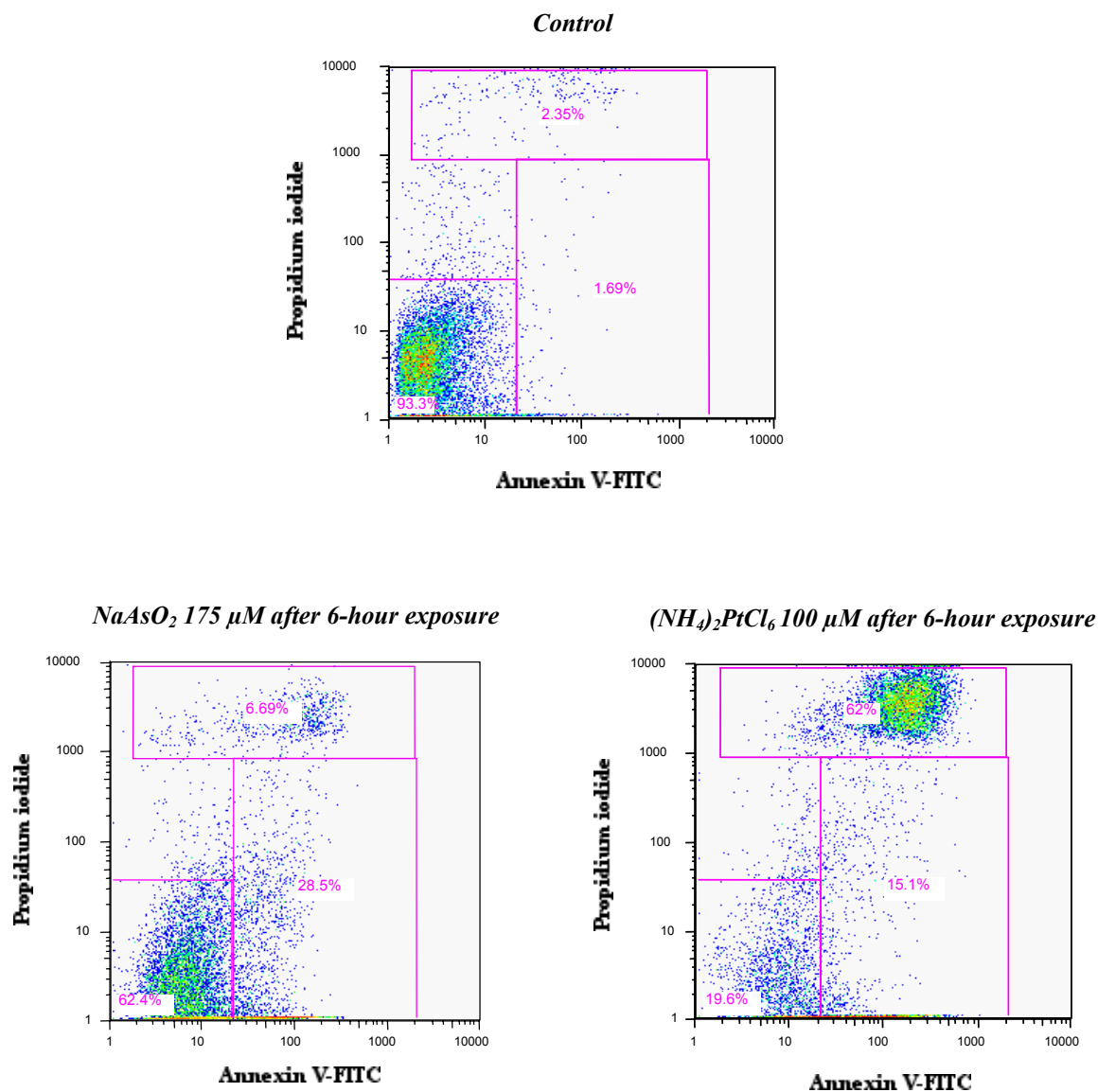
Moreover, figure 9.1b reports some examples of the cytograms obtained after 6-hour exposure of Balb/3T3 cells to 175  $\mu$ M NaAsO<sub>2</sub> and 100  $\mu$ M (NH<sub>4</sub>)<sub>2</sub>PtCl<sub>6</sub>.

*Figure 9.1: Annexin V/PI assay by flow cytometry*

*a) Uncompensated and compensated fluorescence*



**b) Cytograms obtained after exposure of Balb/3T3 cells to As- and Pt-compounds using the fluorescence compensation protocol**



In order to assure the reliability and particularly the reproducibility of the performed experiments, data referring to the controls of each metal compound tested were compared. No significant variations were found among all tests carried out after different exposure times as well as in different days.

## 9.2 Induction of Caspase-3 Activity

For detecting and measuring caspase-3 activity in the Balb/3T3 cells, the PhiPhiLux G<sub>1</sub>D<sub>2</sub> Kit (code n° AK-304R1G3 – MBL, Japan) was used. This method is based on a fluorogenic substrate for caspase-3 and caspase-3-like proteases in living cells. The substrate molecule contains two fluorophores (rhodamines) and assumes loop conformations highly specific for caspase-3, which, if activated, cleaves the amino acid sequence of the fluorogenic substrate:



In the intact peptide of the substrate molecule the cyan coloured fluorophores form a ground-state dimer. Peptide cleavage abolishes this dye-dye interaction and results in an increase in fluorescence and significant absorption changes. Moreover, the substrate is rather hydrophobic and that allows it to pass through the intact membrane. Once the substrate is cleaved, it is more hydrophilic, so it cannot go back through the lipid membrane easily and it stays in the cells.

In the present investigation, 200000 cells/well were seeded in 6-well culture dish (FALCON) adding 3 ml/well of culture medium. After 24 h from cell seeding, the medium was replaced by 3 ml/well of testing solution. Only in the negative control non-treated medium was added. Each metal compound tested was analysed at specific concentrations and exposure times (6, 12 or 24 h).

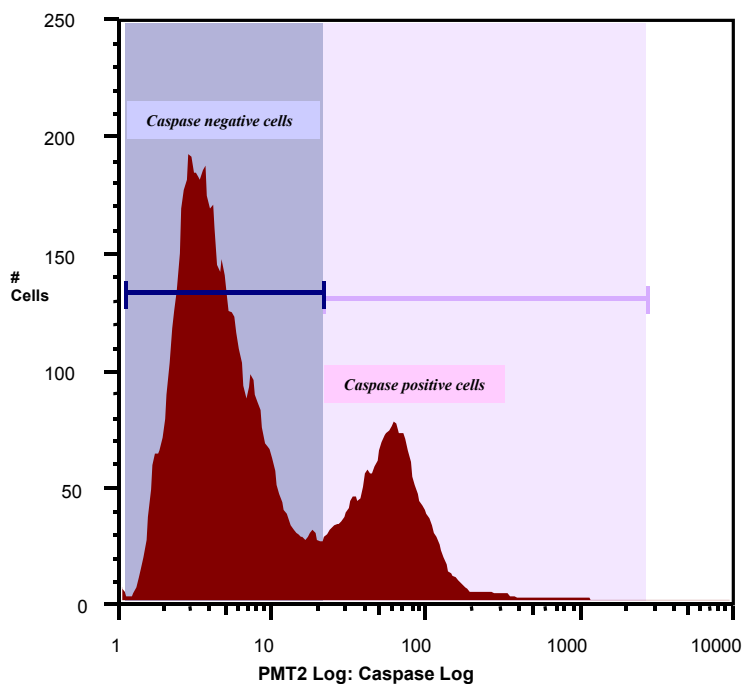
For sample preparation the adopted precautions were the same as those described in Section 9.1. The protocol used was in accordance with the recommendations supplied by kit:

at the end of the exposure time attached and suspended cells were collected using trypsin-EDTA and washed out the testing solution with PBS. Each sample was incubated with the provided substrate solution and then washed with the supplied dilution buffer. Afterwards, each cell sample was analysed by flow cytometry (Epics Elite ESP, COULTER).

The cleaved substrate had the following fluorescence peak characteristics:  $\lambda_{ex} = 505 \text{ nm}$  and  $\lambda_{em} = 530 \text{ nm}$ . (The fluorescence of the precleaved profluorescent protease substrate is not completely quenched). The cleaved substrate was collected in PMT2 (green) and PI in PMT4 (Red) upon excitation at 488 nm.

A positive control was used to set up the caspase positive (+) gates (Figure 9.2). A histogram was displayed on the gated sample of green log fluorescence (the cleaved substrate), and negative (living) and positive (apoptosis – caspase-3 and caspase-3-like activities).

Figure 9.2: Graphic representation of caspase negative and caspase positive cells



During the performance of the present assay, the analysis of the scatters related to each sample was fundamental to understand data that did not report in a clear fashion the percentages corresponding to the caspase negative and the caspase positive populations. In order to explain better this problematic, we have to consider the information provided by scattered light. In fact, morphologically, rapid cell shrinkage and an increase in cell granularity are the most obvious changes associated with

apoptosis. These changes allow populations of apoptotic and viable cells to be distinguished from each other rapidly and quantitatively using the light-scatter properties on a flow cytometer. Indeed intersection of a cell with the light of the laser beam in a flow cytometer results in light scatter. Analysis of the scattered light provides information about cell size and structure. The forward light scatter correlates with cell size and the scattered light measured at a right angle ( $90^\circ$ ) to the laser beam (“side scatter”) correlates with granularity, refractiveness, and the ability of the intercellular structures to reflect the light. The cell’s ability to scatter light is altered during cell death, reflecting the morphological changes, such as cell swelling or shrinkage, rupture of the plasma membrane, chromatin condensation, nuclear fragmentation, and shedding of apoptotic bodies. The necrotic mode of cell death is characterised by a rapid initial increase in the cell’s ability to scatter light simultaneously in the forward and right angle direction, which, in all probability, is a reflection of cell swelling (*Gorczyca W. et al., 1998; Gorman A. et al., 1996*). During apoptosis, on the other hand, the decrease in forward scatter is not initially paralleled by a decrease in right-angle scatter. Actually, an increase in right-angle scatter, simultaneous with a decrease in forward scatter, can be observed in some cell system. In later stages of apoptosis, however, the intensity of light scattered both forward and at a right-angle decreases (*Darzynkiewicz Z. and Li X., 1996*).

In this context, a scatter plot was set up of Forward versus Side Scatter. This was used to gate out debris. During the analysis of the results, the corresponding scatter was always taken into account. Doing so, when a large amount of cells showed a dramatic decrease in SS and FS compared to the control, they were classified as a necrotic population or cells in late stages of apoptosis (strongly damaged cells). Therefore, the related treatment was considered not suitable to provide useful information to the study (see also Section 16.2).

Figure 9.3 shows two distinct cases: *i*) the treatment  $350 \mu\text{M Na}_2\text{CrO}_4 \cdot 4\text{H}_2\text{O}$  after 6-hour exposure shows cells with decreased values of SS and FS. However, this is not a strong decrease (the cells do not appear strongly damaged) (Figure 9.3*a*);

*ii*) most of cells appear strongly damaged as shown by the treatment at  $200 \mu\text{M cis-Pt}$  after 12-hour exposure that identifies low values of SS and FS. When compared with the control, most of these cells represent a population very similar to debris. Therefore, in the related graphic for measuring the caspase-3 activity it is not possible to

distinguish the peak of caspase negative cells and the peak of caspase positive cells (Figure 9.3b). This means that the detection limit of the kit was reached. Indeed, the use of the PhiPhiLux kit is optimal with samples containing a low percentage of necrotic cells.

Figure 9.3: Analysis of scatters related to induction of caspase-3 activity

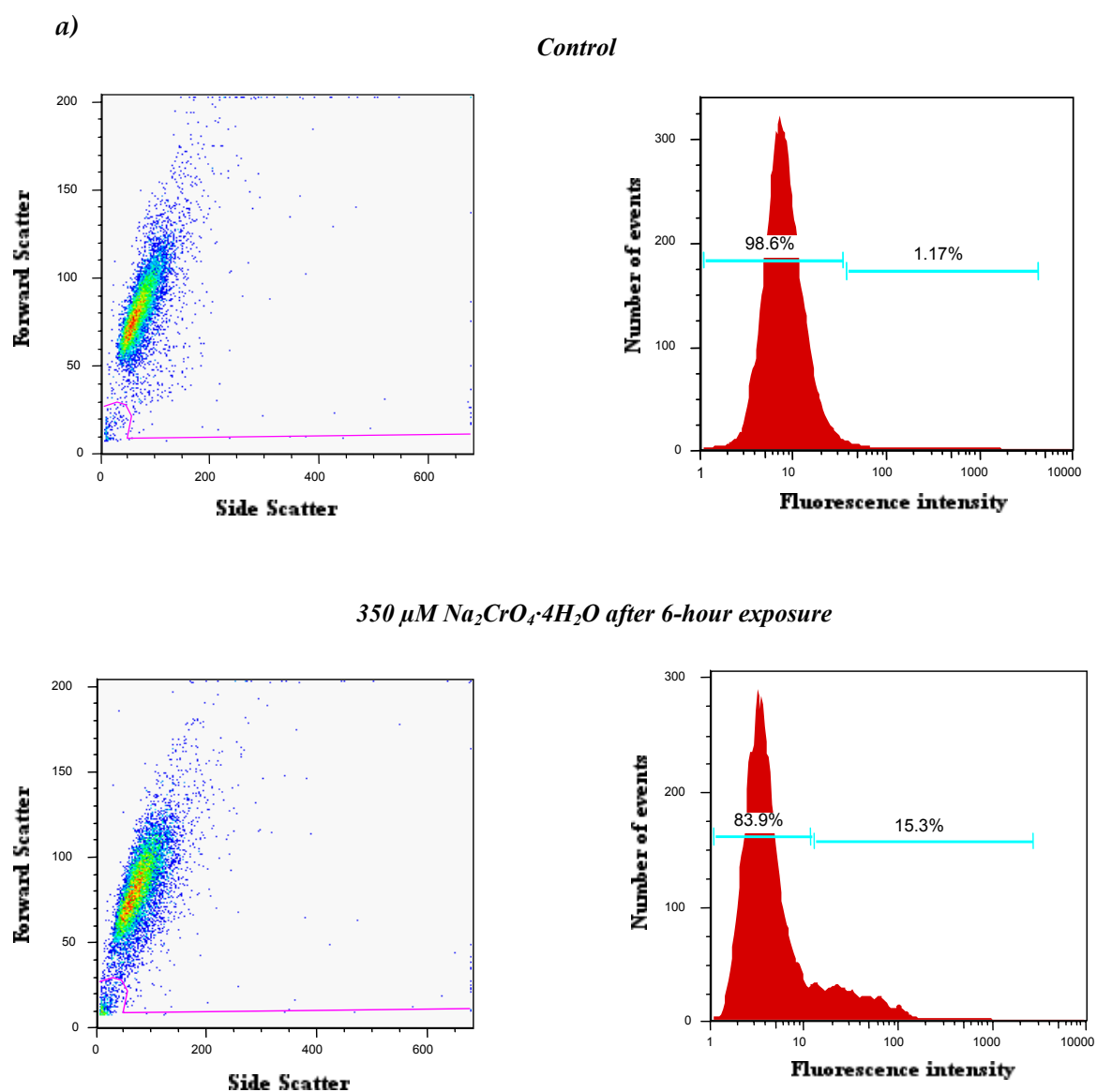
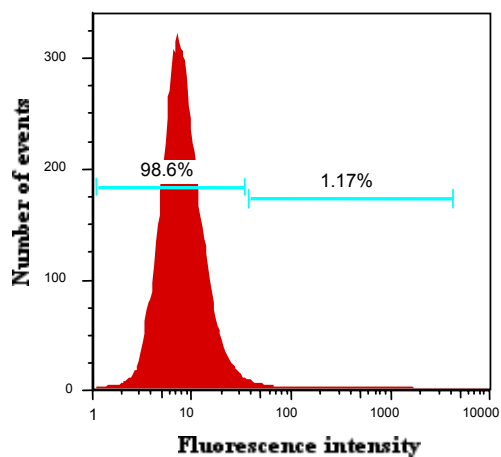
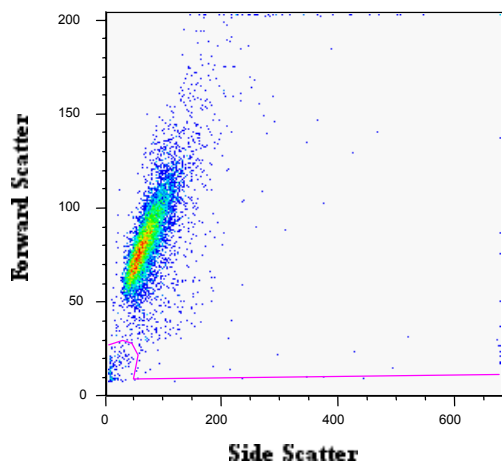


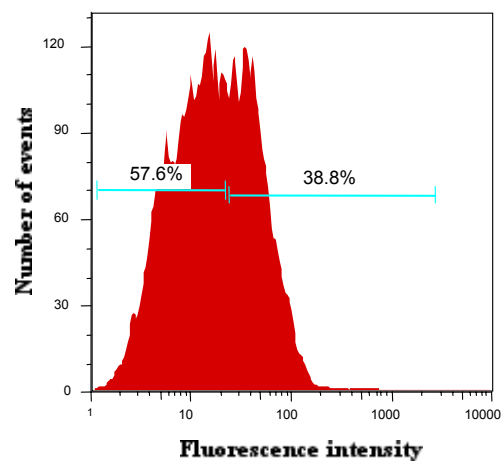
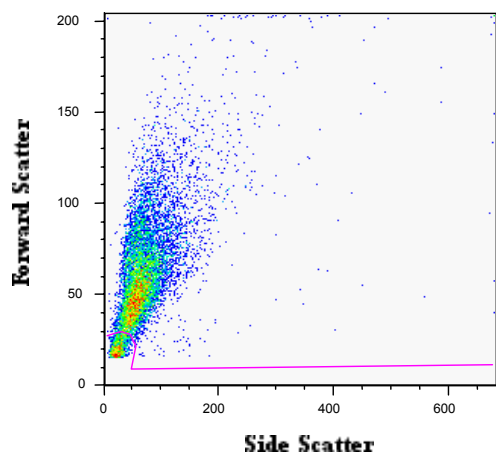
Figure 9.3: Continued

b)

Control



200  $\mu$ M cis-Pt after 12-hour exposure



### 9.3 Nuclear DNA Fragmentation Assay

Apoptotic cells undergo many distinct morphological and biochemical changes, including fragmentation of nuclear DNA. During apoptosis cellular endonucleases cleave nuclear DNA between nucleosomes, producing a mixture of DNA fragments, whose length varies in multiples of 180 to 200 bp (*Schwartzam R.A. and Cidlowski J.A., 1993*).

The ApoAlert DNA Fragmentation Assay Kit (code n° PT3137-1, CLONTECH, Palo Alto, USA) was used to detect DNA strand breaks in cells undergoing apoptosis via a fluorescence assay. The assay is based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (or TUNEL). TdT catalyses incorporation of fluorescein-dUTP at the free 3'-hydroxyl ends of fragmented DNA; fluorescein-labeled DNA can be detected via fluorescence microscopy or flow cytometry.

In this investigation 35000 cells/well were seeded in a plastic 8-well chamber slide (NUNC) adding 0.5 ml in each well. Every treatment was performed in duplicate. After 24 h of incubation the Balb/3T3 cells were treated with 0.5 ml of testing solution at selected concentrations and exposure times (6 or 12 hours), except for the negative and the positive controls, where non-treated medium was added. After the treatment the cells were not detached from the chamber slides but prepared for the analysis as attached cells. In order to avoid the loss of cells eventually in suspension, after the exposure time the medium of each well was collected in individual tubes and every sample was prepared with cytospin (Cytospin 2 – SHANDON, Milano, Italy). This procedure entailed the centrifugation of cell samples twice (700 rpm, 5 minutes) in order firstly to remove all culture medium and then to wash the cells with 5 ml PBS. To each cell pellet 100 µl PBS were added. The cells were gently resuspended, and then load on the slides previously prepared for the cytospin. The samples run 10 minutes at 1000 rpm.

Both chamber slides and cytospin slides were prepared for the analysis in accordance with the instructions supplied by the kit:



cells were fixed with fresh 4% formaldehyde/PBS solution and then permeabilised with 0.2% Triton X-100/PBS solution. (Note: the positive control was prepared by treating normal cells with 1 µg/ml DNase I in a convenient DNase I buffer). After cell washing, the samples were incubated with the TdT incubation buffer. This mixture was TdT-minus only for the negative control. After several washings, the cells were incubated in the dark with 0.1 µg/µl DNase-free RNase in PBS and then with 0.5 µg/ml PI in PBS. To each sample a drop of Anti-Fade solution (MOLECULAR PROBES, Leiden, The Netherlands) was added. Afterwards, each cell sample was analysed by confocal microscopy.

Regarding confocal microscopy, the used Laser Scanning Confocal System was a Bio-Rad Radiance 200 MP (BIO-RAD, Milano, Italy) in combination with a Nikon Eclipse TE 300 inverted microscope (NIKON, Firenze, Italy).

Confocal microscopy is a technique where the specimen is pointwise illuminated by a focused beam of light (usually from a laser). An image is recorded by scanning the beam of light over the specimen, and the reflected or fluorescent light from the specimen is focused onto a small detector aperture. This combination of point illumination and point detection results in a unique "optical sectioning" capability. This optical sectioning makes it possible to record images of thin layers within the specimen without cutting it into slices. By collecting a "stack" of such images from different depths, it is possible to display and quantify the three-dimensional structure of a specimen (*Dobrucki J. and Darzynkiewicz Z., 2001*).

#### **9.4 Hoechst 33342/PI Assay**

The Vibrant Apoptosis Assay Kit (code n°: V-13244 – MOLECULAR PROBES) was used as a convenient assay for apoptosis based upon fluorescence detection of the compacted state of the chromatin in apoptotic cells. The Hoechst 33342 dye ( $\lambda_{\text{ex}} = 350$  nm,  $\lambda_{\text{em}} = 461$  nm, when bound to DNA) stains the condensed chromatin of apoptotic cells more brightly than the chromatin of normal cells; the red fluorescent PI dye ( $\lambda_{\text{ex}} =$

535 nm,  $\lambda_{em} = 617$  nm, when bound to DNA) is permeant only to dead cells.

In the present investigation 200000 cells/well were seeded in 6-well culture dishes (FALCON) and then incubated 24 h. After this time, the Balb/3T3 cells were treated at a selected concentration and exposure time for each metal compound tested. After exposure, the cells of every treatment were collected using the precautions for cell handling as already explained in Section 9.1. The culture medium was removed; the cells were washed with PBS and from this suspension 200000 cells/ml were transferred into a new tube. This 1 ml of cells was incubated in the dark with Hoechst 33342 and PI in accordance with the protocol provided by the kit (Note: the PI concentration was decreased to 0.25  $\mu\text{g/ml}$ ). After this staining, the cells were washed with PBS, then cell pellet was resuspended in 1 ml PBS and 100  $\mu\text{l}$  of this solution were cytospun (500 rpm, 10 minutes). The resultant slides were analysed with a fluorescence microscope (IX 70, OLYMPUS) using two separated filters.

## ***10 Biotransformation and Interaction of Metal Compounds in Culture Medium and Cellular Lysate***

The collaboration with the University of Torino and the Bioindustry Park of Ivrea (Italy) has entailed the application of a spectroscopic method: the High Field Nuclear Magnetic Resonance (NMR) spectroscopy. This is a powerful and theoretical complex analytical tool to investigate the chemical environment of specific nuclei of atoms (<http://www.chemguide.co.uk>; <http://www.shu.ac.uk>).

Two kinds of samples were analysed: culture medium and cellular cytosol.

***Culture medium.*** Culture medium was added to the required reagents in order to be treated with metal compounds in presence and in absence of a specific percentage of proper serum and analysed by NMR. Table 10.I shows the culture media considered.

**Table 10.I: Culture media analysed by NMR spectroscopy**

Culture medium and reagents	Appropriate serum
DMEM Low Glucose (EURO CLONE) [with: 2.5 µg/ml amphotericin B; 4 mM L-glutamine; 30 U/ml penicillin/ 30 µg/ml streptomycin]	10% Foetal Clone III (HY CLONE)
DMEM High Glucose (EURO CLONE) [with: 2.5 µg/ml amphotericin B; 4 mM L-glutamine; 30 U/ml penicillin/ 30 µg/ml streptomycin]	10% Foetal Clone II (HY CLONE)
MEM (EURO CLONE) [with: 5 ml non-essential amino acids solution 100X; 4 mM L-glutamine; 30 U/ml penicillin/ 30 µg/ml streptomycin]	20% Foetal Bovine Serum (Canada FDA approved) (EURO CLONE)
RPMI 1640 (GIBCO) [with: 0.34% genemycin 418; 0.34% hygromycin B; 0.23% tetracyclin; 30 U/ml penicillin/ 30 µg/ml streptomycin]	10% Horse Serum (BIOCHROM, Berlin, Germany)

Once added with the proper reagents (Table 10.I), each culture medium was treated with a selected metal compound. Referring to the metal compounds tested at 1 mM and at 5 mM, these concentrations were reached dissolving the salt directly in the culture medium. With regard to the metal compounds tested at 0.1 mM, a 25 mM solution in D<sub>2</sub>O was initially prepared. Then, the final concentration required for the treatment was reached adding a proper amount into the culture medium.

To all samples a drop of acetone was added for calibration.

**Cellular lysate.** Figure 10.1 reports the protocol used for preparing cellular lysate of the Balb/3T3 cells exposed 72 hours to selected metal compounds.

In order to exclude any problem of osmolarity eventually caused by using PBS as buffer, the pellet obtained after centrifugation at 4°C and 12000 rpm was treated with a solution of NaOH 0.2N and 1% SDS. After few minutes, nucleus denaturation was evident. This excluded any damage for nuclear organelles at least.

**Figure 10.1: Preparation of cellular lysate for NMR analysis**

Seed 1,500,000 cells/dish in 150 x 25 mm culture dish (COSTAR) adding 25 ml/dish of culture medium. Incubate 24 h.



Replace medium with testing solution or fresh culture medium for the negative control. Incubate 72 h.



Transfer each testing solution and the non-treated medium of the control into individual tubes. Centrifuge 5 minutes at 1000 rpm, then stored each solution at -20°C using new tubes.



Trypsinise cells with 1.5 ml/dish of 0.5 g/l trypsin solution without EDTA (SIGMA). Count cells and centrifuge them twice (1000 rpm, 5 min.) in order to wash out the culture medium.



Suspend cells in 0.5 ml PBS and homogenise them maintaining the potter in ice.



Centrifuge the homogenate 10 minutes at 12000 rpm at 4°C.



Ultracentrifuge the supernatant 90 minutes at 100000 x g at 4°C.



Gradually freeze the lysate in a cryobox and then store at -80°C.

NMR analysis of culture media and cellular lysates was carried out according to the following protocol: to 550 µl of each sample 50 µl of D<sub>2</sub>O were added in order to detect a deuterium signal intense enough for the lock. This solution was transferred in a 5 mm NMR tube, which was inserted into the magnet. For each sample the <sup>1</sup>H-NMR spectra were acquired using a NMR Bruker Avance600 spectrometer (BRUKER, Kalsruhe, Germany) that worked with an applied magnetic field 14.1 T (600.13 MHz proton Larmor frequency) at the specific pulse sequence p3919gp (water suppression using 3-9-19 sequence with magnetic field gradients) using the following parameters for data acquisition: 32 K data points with 128 increments for the analysis of culture media

and 512 increments for the analysis of cellular lysates; D1 = 3.5 sec; gradients on z GP1 = GP2 = 20%. The used probe was a TXI 600 SB H-C/N-D-05 Z grd, 5 mm; inverse probe at fixed channels with triple resonance ( $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ) and gradient on Z axis, 5 mm tubes and thermocouple T. Profiles of the culture media were recorded at 298 K, while the profiles of the cellular lysates at 277 K. The resultant data were processed using a Bruker XWIN-NMR version 2.6 software.

## 11 *Statistical Analysis and Data Presentation*

Results concerning cytotoxicity and metabolic studies are expressed as mean  $\pm$  SEM (Standard Error of Mean) of three independent experiments. Values are presented as percentage of the corresponding controls (set at 100%) receiving no test metal compound.

For the CFE experiments, the data were examined with two-way analysis of variance (ANOVA) without replication, to evaluate the reproducibility among experiments performed at different days with a threshold for significance of  $p < 0.05$ .

The dose-response curves were obtained by plotting the experimental data in a semi-logarithmic plot.

The  $\text{IC}_{50}$  (concentration causing 50% reduction in growth compared to the control) value was first calculated from the graph of the dose-response curve and then it was confirmed by applying the Reed and Munch formula (*Reed L.J. and Munch H.A., 1938*). In case the 50% inhibitory effect was not reached, an extrapolated  $\text{IC}_{50}$  was obtained by the USEPA approach (*USEPA, 1991*), which is based on two assumptions for a Colony Forming Efficiency dose-response curve: a) the effect is linearly proportional over the whole concentration range; and b) the linear dose-response curve ( $y = ax + b$ ) is defined by two points, the first one (0; 100) being the viability at zero concentration ( $b$  in the formula), and the second ( $x_1$ ;  $y_1$ ) indicating the measured viability ( $y_1$ ) at the maximum concentration tested ( $x_1$ ).

The  $\text{IC}_{50}$  [ $x_2$  value corresponding to 50% viability ( $y_2$ )] is then derived from the following ratio:

$$(y_1 - b) : (y_2 - b) = x_1 : x_2$$

## *Materials and methods*

Post-hoc multiple comparisons were performed by the Tukey and the Dunnett tests after the analysis of variance (ANOVA).

For the calculation of the MTT results, the optical density of wells was normalised setting the control at 100%. The data shown are mean  $\pm$  SEM values from three independent experiments.