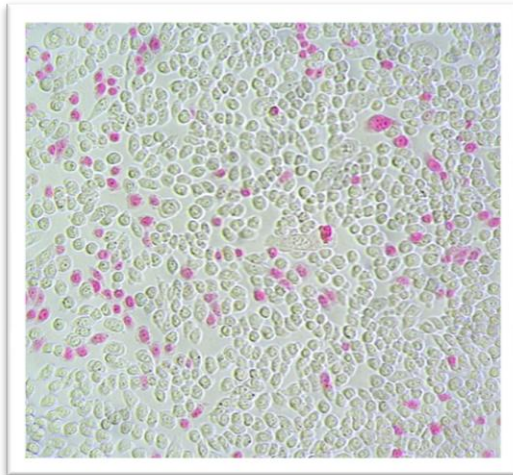


WITH
IMAGEJ
PROTOCOL

APOPercentageTM **Apoptosis** **Assay**



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Analytical Digital Photomicroscopy Protocol

Example given is for the 96-well format, however, the assay may be carried out using 4, 6, 12, 24 and 48-well plates, or microscope chamber slides.

1. Seed a 96-well plate with 2×10^4 cells - 5×10^4 cells/well in 200 μ l appropriate culture medium and incubate the cells at 37°C/5% CO₂, until confluence is reached (~24h).

RB	RB	RB	S	S	S	S	S	S	S	S	S	S
-veC	-veC	-veC	S	S	S	S	S	S	S	S	S	S
+veC	+veC	+veC	S	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S	S	S	S

2. Prepare dilutions of test apoptotic agent(s) at selected concentrations using the suggested layout above. Controls (-ve & +ve) should be included with each experiment.

3. Make up double quantity of Reagent A. Use half the volume to prepare Reagent B.

	Reagent A (100 μ l/well)	Reagent B (100 μ l/well)
Reagent Blank (RB)	Culture medium / serum	Reagent A
Negative Control (-ve C) (0% apoptosis)	Culture medium / serum	Reagent A + 5% ν , dye
Positive Control (+ve C) (100% apoptosis)	Culture medium / serum + reference apoptotic agent	Reagent A + 5% ν , dye
Test Samples (S) (> 0%, <100%)	Culture medium / serum + test apoptotic agent	Reagent A + 5% ν , dye

4. Remove the culture medium from each well of the incubated plate and add 100 μ l of Reagent A, supplemented ν , with serum (if required by the cells), to all wells.

5. Incubation time for apoptotic inducer/inhibitor will depend on apoptotic agent used. 30 min before this time period is reached remove Reagent A. Immediately replace with 100 μ l Reagent B and incubate for the remaining 30 min, at 37°C/5% CO₂.

6. Remove Reagent B from each well using a pipette. Gently wash the cells twice with 200 μ l/well PBS to remove non-cell bound dye. (NOTE: Careful pipetting is advised as some apoptotic agents can cause detachment and loss of cells).

7. Add sufficient PBS to cover the cells and view immediately with an inverted microscope fitted with a digital camera (microscope/camera settings see page 5).

8. Photograph three representative areas from each well, at 100x magnification.

9. Transfer photomicrographs to a computer and count the stained cell area present in each photomicrograph, using ImageJ software suite.

FOR IMAGEJ SOFTWARE USE GO TO PAGE 5

Colorimetric Protocol

1. Seed a **24-well** tissue culture plate with 5×10^4 cells/well in 500µl culture medium and incubate the cells at 37°C/5% CO₂, until confluence is reached (~ 24h).

Reagent Blank	Reagent Blank	Negative Control	Negative Control	Positive Control	Positive Control
Sample 1	Sample 1	Sample 4	Sample 4	Sample 7	Sample 7
Sample 2	Sample 2	Sample 5	Sample 5	Sample 8	Sample 8
Sample 3	Sample 3	Sample 6	Sample 6	Sample 9	Sample 9

2. Prepare dilutions of test apoptotic agent(s) at selected concentrations using the suggested layout above. Controls (-ve & +ve) should be included with each experiment.
3. Make up double quantity of Reagent A. Use half the volume to prepare Reagent B.

	Reagent A (500µl/well)	Reagent B (500µl/well)
Reagent Blank	Culture medium / serum	Reagent A
Negative Control (0% apoptosis)	Culture medium / serum	Reagent A + 5% ^v / _v dye
Positive Control (100% apoptosis)	Culture medium / serum + reference apoptotic agent	Reagent A + 5% ^v / _v dye
Test Samples (> 0%, <100%)	Culture medium / serum + test apoptotic agent	Reagent A + 5% ^v / _v dye

4. Remove the culture medium from each well of the incubated plate and add 500µl of Reagent A, supplemented ^v/_v with serum (if required by the cells), to all wells.
5. Incubation time for apoptotic inducer/inhibitor will depend on apoptotic agent used. 30 min before this time period is reached remove Reagent A. Immediately replace with 500µl Reagent B and incubate for the remaining 30 min, at 37°C/5% CO₂.
6. Remove Reagent B from each well using a pipette. Gently wash the cells twice with 1000µl/well PBS to remove non-cell bound dye. (NOTE: Careful pipetting is advised as some apoptotic agents can cause detachment and loss of cells).
7. Add trypsin (50µl) to each well and incubate for 10 minutes at 37°C/5% CO₂. Tap the plate gently by hand after 5 minutes and again after 10 minutes to detach cells from the plastic, cell culture treated surface.
8. Now add 200µl Dye Release Reagent to each well and shake plate for 10minutes.
9. Transfer contents of each well (250µl) to a 96 well flat bottom plate and read absorbance at 550nm, (or blue-green filter), using a microplate reader. Bubbles in wells affect results, burst with clean pin or transfer 200µl instead of 250µl to microplate.

FOR ASSAY CALCULATIONS GO TO PAGE 4

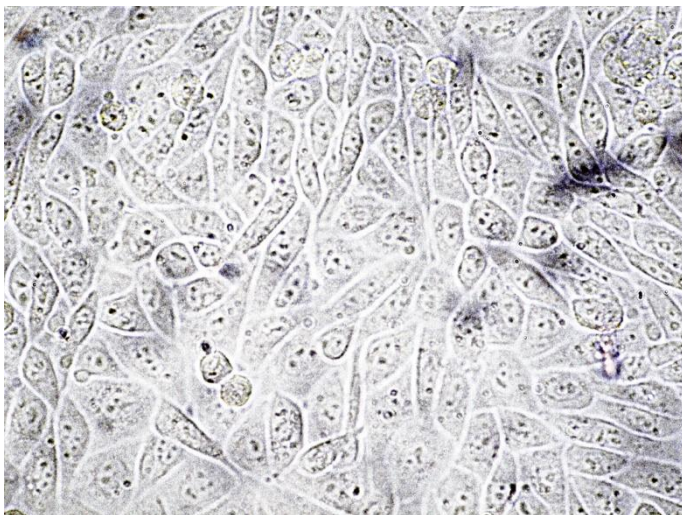


Fig. 4a Photomicrograph Showing Uninduced CHO Cells Treated with APOPercentage Dye, (magnification x400).

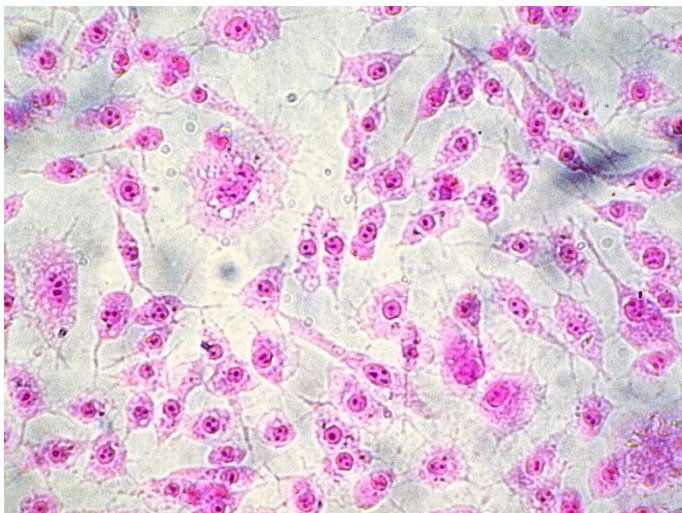


Fig. 4b Photomicrograph Showing CHO Cells Induced with Hydrogen Peroxide (10mM) and Treated with APOPercentage Dye, (magnification x400).

APOPercentage™

Apoptosis Assay

The assay has been designed for research work only.

Handle the APOPercentage Assay using Good Laboratory Practice.

TECHNICAL INFORMATION

GENERAL ASSAY PROTOCOLS

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ASSAY MANUAL

Intended Applications

The APOPercentage™ Apoptosis Assay is a detection and measurement system to monitor the occurrence of apoptosis in mammalian, anchorage-dependent cells during *in vitro* culture.

The assay uses a dye that is selectively imported by cells that are undergoing apoptosis. Necrotic cells cannot retain the dye and therefore are not stained.

Protocol and Format

Two different protocols are available and described on the inside covers of this manual. The approach adopted will depend on the user's choice of quantification method.

(a) Colorimetry protocol - The dye that accumulates in 30 minutes within labeled cells is released into solution and the concentration of released intracellular dye measured using a microplate colorimeter.

(b) Analytical Digital Photomicroscopy protocol - The dye used has a purple-red colour, which allows detection of apoptosis with a conventional inverted microscope. Photomicrograph images obtained are transferred to a computer where the stained area is counted in pixels using software downloadable from the internet.

This manual describes the colorimetric protocol in a 24-well plate format and the Analytical Digital Photomicroscopy protocol in a 96 well plate. Alternative microwell plates, microscope chamber slides and T-flasks are also suitable for use with the assay. If using a 96 well plate with the colorimetric method it is recommended that the contents of 3 wells be pooled before reading results in the microplate reader to provide a detectable absorbance.

APOPercentage Kit Pack Sizes and Storage Conditions

Standard Assay Kit Product Code: A1000 (4 x 24 wells or 6 x 96 wells; microwell format). All components are stable for up to one year unopened when stored at 25°C. Store at 4°C once opened and use within 30 days.

Assay Kit Components

1. APOPercentage Dye (5ml)

The dye is dissolved in a phosphate buffered saline (PBS) solution, pH 7.4, prepared using pyrogen-free water. The dye solution was prepared by filtering the dye solution through a 0.2µm filter into sterile sealed glass ampoules.

For assay use: dye reagent aliquots should be removed from this vial using a sterile needle and syringe (1 ml or 2 ml capacity is suitable). A dye solution that becomes turbid, or where particles appear, should be discarded as sterility has possibly been compromised.

2. APOPercentage Dye Release Reagent (120ml)

An alkali solution that disrupts the cell membrane and releases intracellular accumulated dye.

3. Phosphate Buffered Saline (PBS), sterile solution (120ml) – starter bottle.

The APOPercentage Assay has been designed for use within the '**Clean Room**' of a Cell Culture Unit.

Other Components required - not supplied

The Colorimetric Protocol Requires

- (a) a working solution of trypsin as used for cell release during cell sub-culturing
- (b) a **Microplate Colorimeter** to measure released dye, (a BMG Labtech Spectrostar Nano Microplate Reader was used in the present work)
- (c) 24 well plates for culturing cells during apoptosis testing
- (d) 96 well plates for use when reading absorbance values

The Analytical Digital Photomicroscopy Protocol Requires

- (a) an inverted stage **microscope with an attached digital camera**
- (b) a **computer with a pre-installed copy of ImageJ** (Java-based image processing program available at <http://rsbweb.nih.gov/ij/>)
- (c) 96 well plates for culturing cells during apoptosis testing.

MODE OF ACTION

The onset of the execution phase of apoptosis has been linked to translocation of phosphatidylserine from the interior to the exterior surface of the mammalian cell membrane, experimentally supported by annexin-V binding to phosphatidylserine. Phosphatidylserine transmembrane movement results in the uptake of the APOPercentage Dye by apoptotic committed cells. This dye uptake continues until *blebbing* occurs. No dye is then released from the now defunct cell, other than that within isolated 'blebs'.

Each apoptotic inducer has an optimum time and concentration to be effective. This optimum time and concentration will be affected by the cell line examined.

ASSAY PREPARATIONS

Choice of Protocol: Choose protocol according to laboratory equipment available. These protocols should be considered as start-up guides only. Seeding cell density for particular cells used and incubation time required for apoptotic agent of choice will need to be optimized.

Format: The APOPercentage™ Assay has been designed for use with anchorage dependent mammalian cells seeded in sterile tissue culture containers. A 24 well plate format has been used as a working example of the assay with the colorimetric protocol and a 96 well plate with the analytical digital photomicroscopy protocol.

Seeding Density: In order to compare experiments carried out on different days the cell seeding density and the time required to obtain a confluent layer should be maintained.

Negative Control: Occurrence of apoptosis within an uninduced primary cell/cell line should be assayed to examine the background frequency of dye labeled cells.

Positive Control: The use of 10mM H₂O₂ for four hours to induce 100% dye labelled cells is recommended. Test results can be expressed as a percentage of this data to display percentage apoptosis.

Reagent Blank: A reagent blank sample should be included in each experiment to take into account the background absorption value due to, for example, Phenol Red and serum if used in culture medium.

Cell Adhesion: If unacceptable cell loss is encountered with test agent(s), consider growing the cells on a gelatin gel covered by culture medium (protocol available from our website).

COLORIMETRIC PROTOCOL EXAMPLE CALCULATIONS

1. Follow protocol on inside back cover of manual to obtain absorption data.
2. Subtract the mean value of reagent blank replicates from test results obtained as shown in table below. (This experiment was carried out with triplicate samples). The data shown was obtained using a BMG Labtech Microplate Reader.

H ₂ O ₂ (mM)	Raw Absorbance Data			Mean Reagent Blank Value	Raw Data Minus Reagent Blank		
0	0.062	0.061	0.058	0.053	0.009	0.008	0.005
0.5	0.066	0.072	0.068		0.013	0.019	0.015
1.0	0.067	0.080	0.071		0.014	0.027	0.018
2.5	0.179	0.187	0.196		0.126	0.134	0.143
5.0	0.226	0.228	0.217		0.173	0.175	0.164
7.5	0.234	0.239	0.247		0.181	0.186	0.194
10.0	0.245	0.255	0.236		0.192	0.202	0.183

3. Plot mean absorbance values \pm standard error of the mean in a bar chart (Fig. 1) or as a percentage of the Positive Control absorbance value.

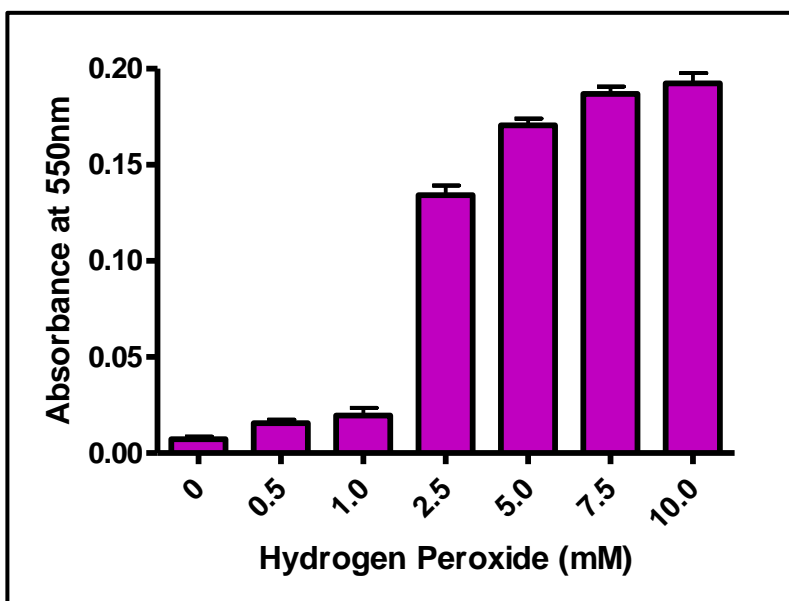


Fig. 1 Colorimetric Quantification.

Graph Showing Effect of Hydrogen Peroxide (0 – 10mM) (4 hours) on CHO Cells. Results expressed as mean absorbance for triplicate wells \pm S.E.M.

(n = 3).

ANALYTICAL DIGITAL PHOTOMICROSCOPY AND IMAGEJ PROTOCOL

Follow the protocol on inside front cover of manual. Count pixel number in photomicrographs using ImageJ software. **ImageJ** software is freely available to download from <http://rsbweb.nih.gov/ij/>.

Camera/Microscope Settings

The white balance on the camera should be set using the negative control well of the microplate to provide a pale gray background. The other settings on the microscope and camera such as iris aperture, brightness, picture quality, resolution, focus and zoom **must** remain constant within each experiment.

At least three photographs should be taken of each well to provide an average pixel count of apoptotic area. All photographs in an experiment should be at a constant magnification. The fields of view selected should be of similar confluence to ensure apoptotic pixel counts are representative.

Once the images are on the computer follow the protocol outlined below to obtain apoptotic pixel count in each photograph.

Use of ImageJ to Quantify APOPercentage Assay Photomicrographs

This protocol outlines the counting of stained cell area against a background of substrate and unstained cells.

1. Open the **ImageJ** program and click **File > Open** from the menu on the **ImageJ** toolbar. The required image can then be opened for analysis.

2a. Click **Image > Adjust > Color Threshold** from the main **ImageJ** toolbar menu. It may be necessary to click the **Original** button at the bottom of the **Color Threshold** window to remove the default threshold values automatically assigned by **ImageJ**.

2b. Hold down the 'shift' key and highlight the pink-coloured area **ONLY** of three or four stained cells on the image (Fig. 2). It is not necessary to highlight the whole cell. Do not include the background colour in your selection. It is important that this step is accurately performed.

2c. Click the **Sample** button at the bottom of the **Color Threshold** window. This will cause the image to change to a **binary image** comprised of cells highlighted in a single colour (black, white or red) against the image background. This image is now ready for analysis.

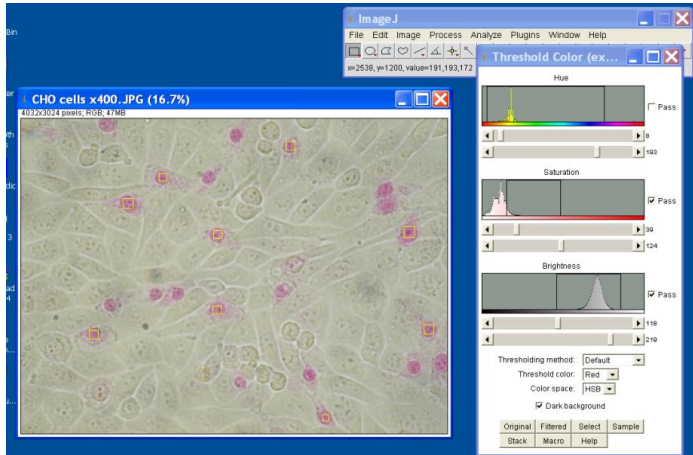


Fig. 2 Screen Shot Showing ImageJ Sample Selection of Dye Labelled Cells.

3a. Select **Analyze > Analyze Particles** from the main **ImageJ** toolbar menu.

3b. In the **Analyze Particles** window set the **Size (pixel²)** to read *25-Infinity*. Set the **Show** menu to 'overlay masks' using the dropdown menu.

3c. Then select the **Summarize**, **Exclude on edges** and **In Situ Show** checkboxes in the **Analyze Particles** window and click the OK button at the bottom of the **Analyze Particles** window.

4. Provided **steps 2 a-b** are accurately performed, the highlighted areas on the image should visually correspond to apoptotic cells labelled with APOPercentage dye. A final composite image will be produced alongside the results table. This image outlines the pixels the software has counted, providing a visual check against the original image that apoptotic cells have indeed been included.

5. Record the total number of pixels. This can be found in the separate '**Summary**' window that opens with a total number of detected cells in a list. This window is updated with each new image analysed.

6. The process can now be repeated for each subsequent image. The **Color Threshold** and **Summary** windows may be left open during the analysis of subsequent images. Image analysis results displayed in the **Summary** window can be saved as an Excel spreadsheet file by clicking **File > Save**.

7. Plot total apoptotic area in pixels as a bar chart (Fig. 3). Results can also be expressed as a percentage of the Positive Control. This permits a comparison with data derived from the colorimetric procedure (see inside back cover of this manual).

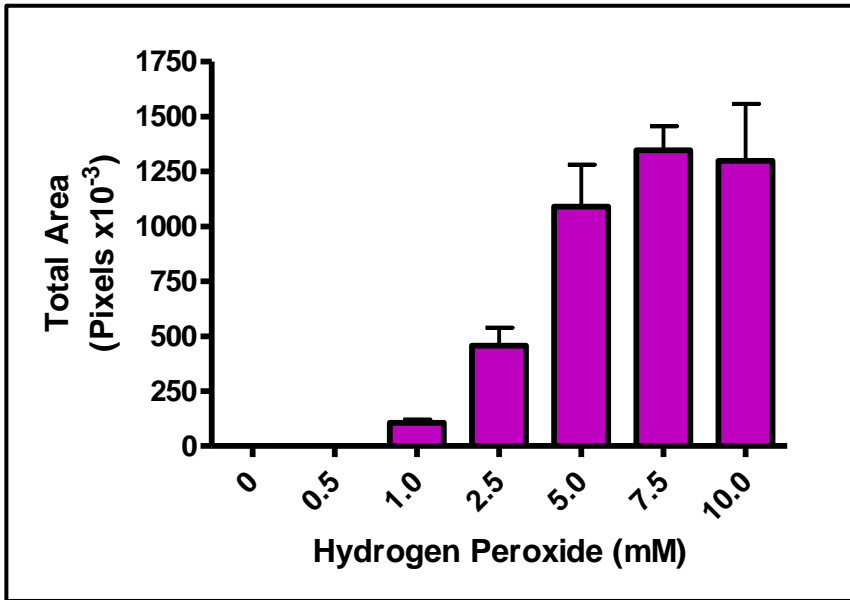


Fig. 3 Analytical Digital Photomicroscopy Quantification.

Graph Showing Effect of Hydrogen Peroxide (0 – 10mM) (4 hours) on CHO Cells. Results expressed as total stained cell area in 3 separate fields of view in triplicate wells \pm S.E.M. (n = 3).

APOPERCENTAGE DYE USED TO STAIN CELLS EXHIBITING APOPTOSIS IN ANCHORAGE-DEPENDENT MAMMALIAN CELLS, USING A RANGE OF APOPTOTIC INDUCERS

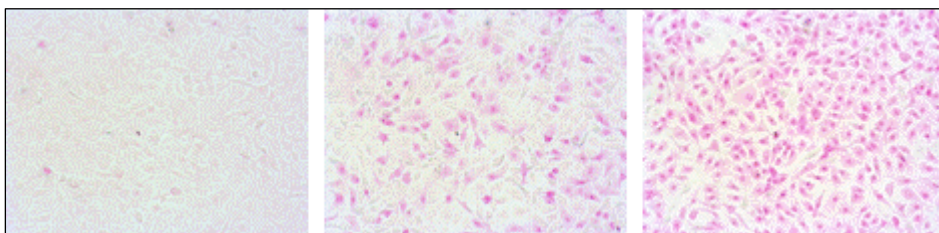
Hydrogen Peroxide (H₂O₂) (5mM)

3T3 Cells

1 HOUR

2 HOURS

3 HOURS

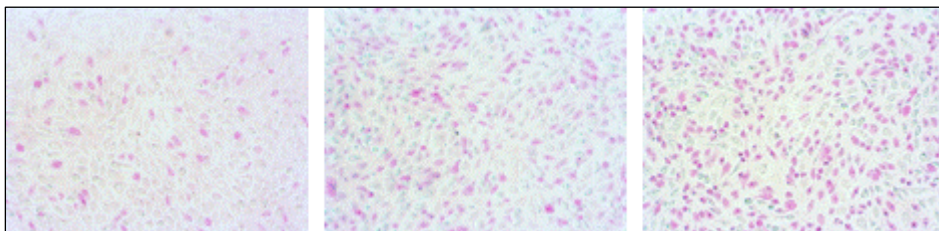


CHO Cells

1 HOUR

2 HOURS

3 HOURS



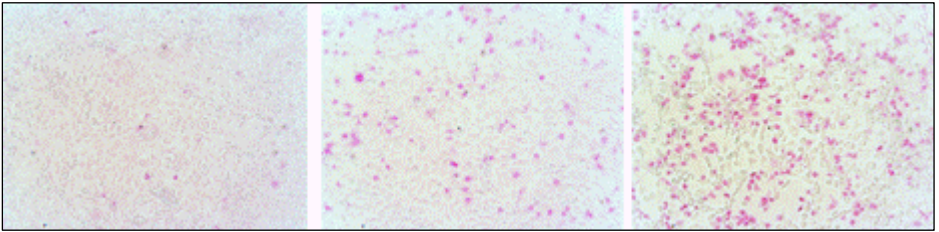
Cyclohexamide (CHX) (10mM)

3T3 Cells

2 HOURS

4 HOURS

6 HOURS

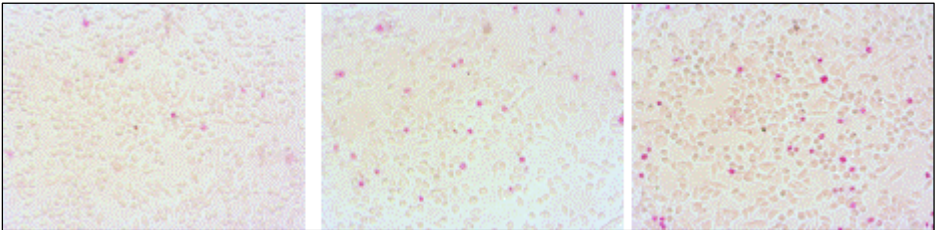


CHO Cells

2 HOURS

4 HOURS

6 HOURS



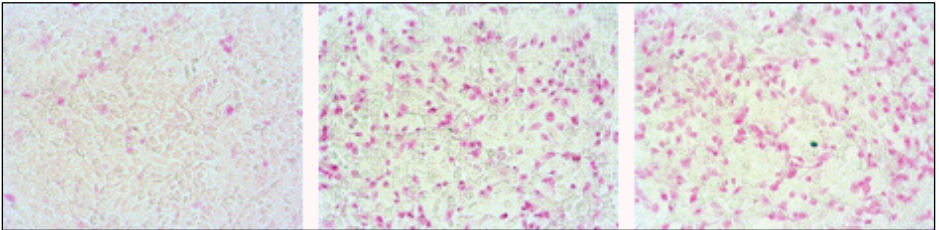
Cisplatin (2mM)

3T3 Cells

2 HOURS

4 HOURS

6 HOURS

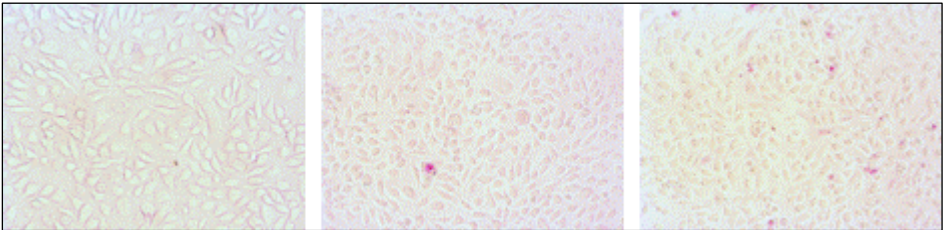


CHO Cells

2 HOURS

4 HOURS

6 HOURS



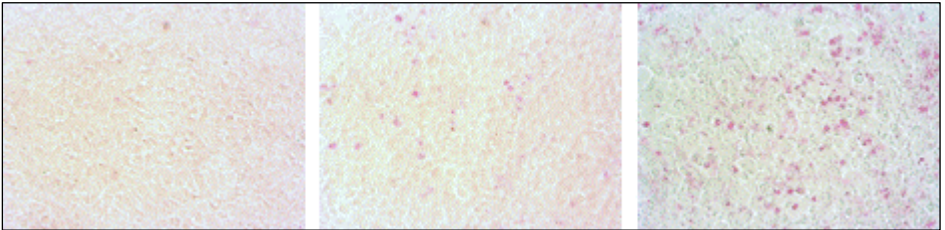
5-Fluorouracil (5-FU) (10mM)

3T3 Cells

6 HOURS

24 HOURS

48 HOURS



CHO Cells

6 HOURS

24 HOURS

48 HOURS

