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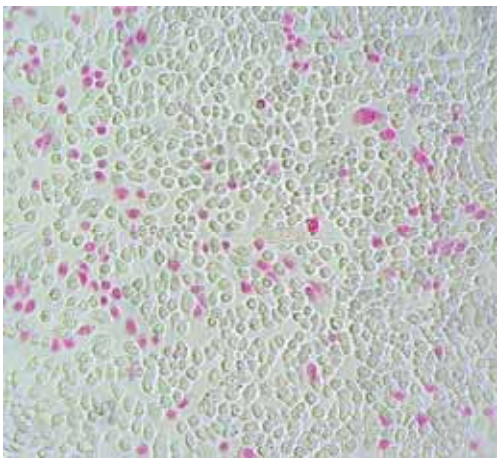
APOPercentage™

Apoptosis

Assay



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APO*Percentage*™ Apoptosis Assay

Time Req: ~45 min **Detection Limit: a single cell**

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. For a discussion of the method see pages 9~13.

Cell treatment and dye labelling:

[1] Add 100µl of 0.4% gelatin (supplied) to each well and allow to settle for at least 10 minutes

[2] Seed selected wells with between $2x10^4$ and $5x10^4$ cells, in 200 µl culture medium

[3] Incubate cells at 37ºC/5% CO_2 until confluence is reached (~ 24 h)

[4] Remove incubation medium and gelatin and rinse cells with fresh medium

[5] Add apoptotic inducer/inhibitor, in 100 µl/well fresh culture medium

[6] 30 mins before the apoptotic inducer/inhibitor incubation time is reached, add 100 µl/well fresh culture medium containing 5 µl APO*Percentage* Dye and the apoptotic inducer/inhibitor to the centre of the well

[7] Incubate for the remaining 30 min of the assay

[8] Syringe off the culture medium/dye mixture, and gently wash the cells twice with 200 µl/well PBS

[9] Transfer immediately to an inverted microscope and obtain at least 3 photomicrographs of a representative area of each well

See inside back cover for apoptosis quantification

APOPercentage™
APOPTOSIS
Assay

TECHNICAL INFORMATION

Contents

The *APOPercentage*™ Assay is supplied
for *in vitro* research work only

Handle the
***APOPercentage*™ Assay Kit**
using
GOOD LABORATORY PRACTICE
Read Manual Before Use

***APOPercentage*™ Manual**

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INTENDED APPLICATIONS

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

Mammalian cells with modified membrane phospholipid composition, such as specialised secretional cells are not recommended test material. Established cell lines (developed from tumour cells) frequently undergo considerable de-differentiation and would be suitable test cells.

The assay uses a dye that is selectively imported by cells that are under going apoptosis.

The most economical experimental design is the 96-well plate format. 6, 12, 24 and 48 well plates, and microscope chamber slides, are also suitable for use with the assay. When larger scale cell populations are to be monitored, T-flasks can be employed.

The cell-dye contact time is 30 minutes. The dye used has a purple-red colour, which allows detection of apoptosis with a conventional inverted microscope, as used in cell culture laboratories (for cell counting and checking cell growth and confluence), at magnifications of x 100 and x 200. With an attached digital camera, photomicrograph images can be obtained and transferred to a computer. These images may then be used for further analysis and quantification by Analytical Digital Photomicroscopy (ADP*) (see Figure 2).

The dye that accumulates within labelled cells can also be released into solution and the concentration of intracellular dye measured using a microplate colorimeter or fluorimeter.

Combining the use of the 96-well format with a 30 min labelling period, results are quickly obtained at a low cost. The 96-well format also permits screening of a wide range of apoptotic agents (see Figures 9-16).

APOP*ercentage*™ ASSAY KIT COMPONENTS

Reagents:

[1] APOP*ercentage* Dye [5 ml];

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 a batch dye solution through a 0.2 µm 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 probably been compromised.

Add dye to complete cell culture medium at a **one in twenty dilution**.

[2] APOP*ercentage* Reference Dye Standard [10 ml];

Prepared at a concentration of 10 µM in Dye Release Reagent.

The reagent is supplied for use as a cell independent reference source.

Cell lines can display considerable differences in the amount of APOP*ercentage* Dye they will accumulate in 30 min.

[3] APOP*ercentage* Dye Release Reagent [150 ml];

An alkali solution, containing sodium hydroxide and surfactants, which is used to disrupt the cell membrane and release intracellular accumulated dye .

[4] Gelatin Matrix forming Solution [100 ml];

A sterile 0.4% w/v gelatin solution in water.

This is an 'optional' reagent, which is recommended when detecting apoptotic levels using the ADP* technique (see page 9). It will also prevent some cell loss, caused by apoptotic inducers.

All of the reagents should be stored at 4°C when not in use.

Other assay kit components:

[5] A sterile, flat bottom, 96-well plate.

[6] A copy of this Manual.

Other components required, but not supplied:

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

ADP* technique — an ordinary inverted stage microscope with a camera (preferably digital) attached and a computer, with GIMP (open-source freeware at www.gimp.org) or Adobe® Photoshop® software installed.

Otherwise a microplate colorimeter or fluorimeter depending on the technique preferred.

REVIEW OF APOPTOSIS ASSAY SYSTEM OPTIONS

The apoptotic process in mammalian cells is a rapid event (2-4 hours). Within this short time span an apparently viable cell can be quietly dismantled, to disappear leaving no visible trace of its former existence.

An apoptosis cascade of activators, effectors and regulators has been identified. This in turn led to a range of apoptosis assays being devised to detect and monitor these events. Some laboratories will employ two distinct assays, one selected to detect early (initiation) apoptotic events, while a second assay will target a later (execution) event. Apoptosis assays, based on methodology, can be classified into four major inter-linked groups:

[1] DNA fragmentation (electrophoresis and nick end labelling, TUNEL)

[2] Apoptotic proteases (fluorescently labelled antibodies to the caspases)

[3] Flow cytometric analysis (FACS, incorporating other group assays)

[4] Membrane alterations (phosphatidylserine flip)

Assay selection considerations:

[1a] DNA fragmentation

Preparation.	Extraction of DNA from lysed cell homogenate, using phenol-chloroform.
Detection.	Following agarose gel electrophoresis.
Sensitivity.	1 x 10^6 cells.
Advantages.	Photographic evidence of large DNA fragments.
Disadvantages.	Can be difficult to produce a nucleosome ladder as further DNA fragmentation can occur during preparation. Necrotic cells also generate DNA fragments.

[1b] Enzymatic end labelling of DNA strand breaks (TUNEL)

Preparation.	Fixation of cells before adding proteolytic enzyme to expose DNA.
Detection.	With terminal transferase (TdT) and labelled UTP – then examined by fluorescence microscopy or FACS.
Sensitivity.	Single cell (or >100 cells by FACS)
Advantages.	Can be completed within 3 hours.
Disadvantages.	Subject to false positives from necrotic cells and risk of high background from some viable cells. Collection times can be critical, too early and DNA fragmentation may not yet be extensive, too late and DNA fragmentation can be excessive.

[2] Apoptotic proteases (caspases)

Preparation.	Some ten caspases (procaspases or active cysteine proteases) are known. Caspases can be detected using labelled antibodies. Detection and measurement of specific caspase activity requires cell lysis and coating of the microwell plate with anti-caspases.
Detection.	Fluorescent labelled substrate.
Sensitivity.	Caspase activity detection usually requires 1 x 10^5 cells.
Advantages.	It is possible to select for individual initiator caspases or execution caspases.
Disadvantages.	Caspase activation does not necessarily imply that apoptosis will occur.

[3] Fluorescence Activated Cell Sorter (FACS)

Preparation	Best used for suspension cell samples.
Detection	Less sensitive than the microscope slide and microplate based versions of Assay Groups 1,2 & 4.
Sensitivity	Requires >100 apoptotic cells to form a discernible cluster in a sample scan.
Advantages	Good option for suspension cells.
Disadvantages	The equipment is designed for cell counting and cell sorting. Excessive physical force, to remove cell clumps, can damage cells.

[4a] Apoptosis membrane alteration: Annexin-V binding

Preparation	Cells are washed free of culture medium using PBS and then incubated with FITC-labelled annexin-V.
Detection	Fluorescent microscopic examination. The membrane of both apoptotic and necrotic cells are dye labelled.
S*ensitivity*	Single cell.
Advantages	Confirms the occurrence of phosphatidylserine flippase in apoptotic cells, and the activity of initiator caspases.
Disadvantages	Does not discriminate between apoptotic and necrotic cells.

[4b] Apoptosis membrane alteration: Dye-uptake bioassay (APOP*ercentage*™ Assay)

Preparation	Add dye to the cell culture medium and return cells to the incubator, for 30 min. Then drain medium from cells and wash with PBS.
Detection	By visible light, using a typical cell culture lab's inverted microscope, (magn x100 or x200). Obtain a photographic record of findings.
Sensitivity	Single apoptotic cell.
Advantages	Quick and easy to use. Assay can also be quantified, using a digital camera or a microplate colorimeter/fluorimeter (96-well format). Necrotic cells cannot retain the dye. Several hundred assays can be performed in one day.
Disadvantages	Limited information available on cell membrane composition.

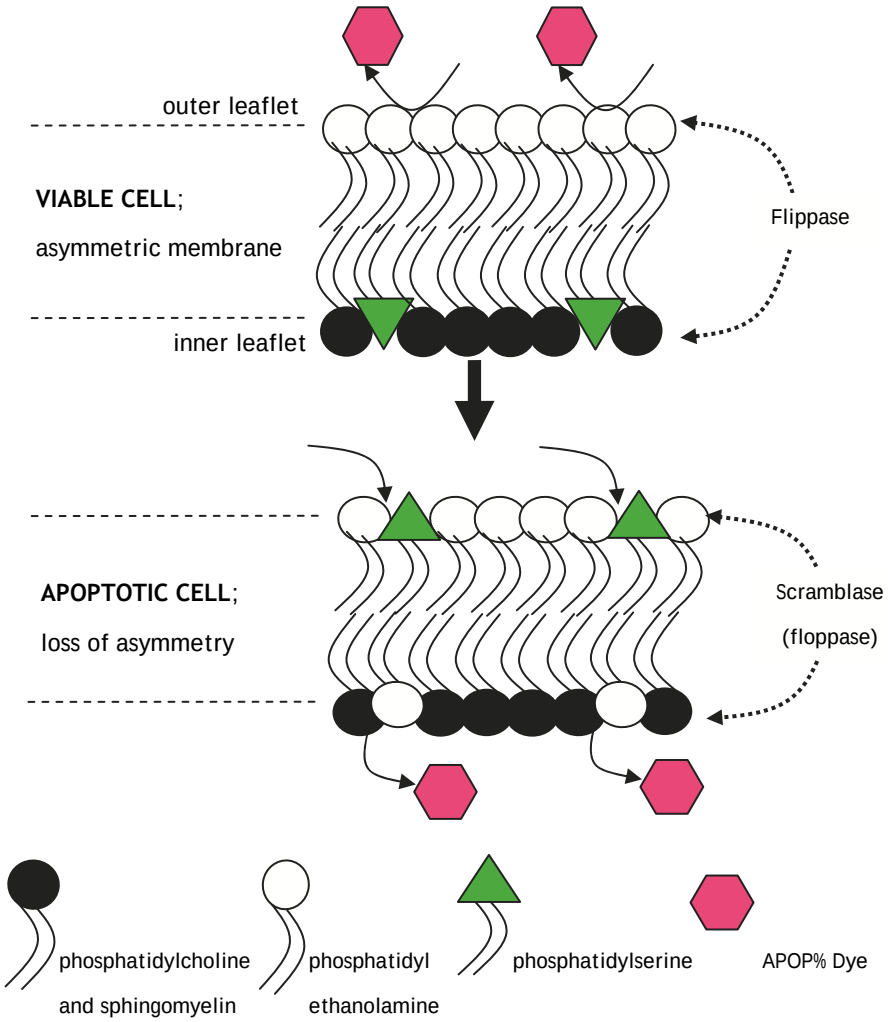


Fig. 3

The asymmetric phospholipid composition of a transformed mammalian cell

The choline phospholipids make up most of the lipid in the extracellular membrane. This organisational composition is essential for the normal functions of a viable cell, including the insertion of protein receptors and transporters between the phospholipid molecules.

Maintaining the asymmetric composition is an energy dependant process involving the activity of enzymes, termed 'flippases'. In ***apoptotic committed*** *cells flippase regulation is either overwhelmed, or is inactivated by the activity of the enzyme 'scramblase' (floppase).*

The transfer of phosphatidylserine to the outside of the membrane permits the transport of the APOPercentage dye into the cell. The uptake of the dye is ***uni-directional****, leading to dye accumulation within the cell. As the cell shrinks in volume, during the apoptotic process, the cell dye content becomes more concentrated.*

THE APOP*ercentage* ASSAY: MODE OF ACTION

To explain the mode of action of the APOP*ercentage*™ Assay requires a closer examination of the mammalian cell membrane. The membrane has been described as a semi-fluid mosaic structure, composed of phospholipids with a diverse group of inserted proteins and some cholesterol. The major components of the membrane are the phospholipids, arranged in the form of a 'bi-layer'; which is ***asymmetric*** in composition, structure and function.

The outer leaflet of the lipid membrane is composed of choline containing phospholipids (phosphatidylcholine and sphingomyelin) that are in contact with the extracellular matrix (ECM) (*in vivo*), or with cell culture medium (*in vitro*). The membrane's inner leaflet is composed of phosphatidyl ethanolamine and phosphatidylserine and is in contact with the cellular cytoplasm. The non-polar, hydrophobic fatty acid tails of the phospholipids of both leaflets make up the interior volume of the membrane, giving the typical bi-layer structure, as seen in EM photomicrographs.

The asymmetric composition of the membrane phospholipids is essential in maintaining a 'viable' cell. The membrane, and its component parts, selectively control the exchange of molecules and generate concentration gradients between the cytoplasm and the ECM. The proteins that regulate these operations fit, asymmetrically, into this membrane and they include a diverse range of carriers (permeases and transporters) and receptors.

To ensure normal transmembrane functions the phospholipids must be maintained in an asymmetric composition. The process is regulated by ***'flippases'***, which catalyse the active transport of aminophospholipids from the outer to inner monolayer (Sprong. *et.al*. [2001] *Nature Reviews Mol. Cell Biol.* **2**, 504-513). In cells undergoing apoptosis, flippase is overwhelmed by the action of another enzyme, termed ***'floppase'*** or ***'scramblase'*** (Zhou. *et.al*. (1997) *J. Biol. Chem.* **272**, 18240- 18244).

The trigger that activates floppase, and inhibits flippase, may be a rapid increase in the intracellular calcium concentration. The net effect of floppase action is a scrambling of the phospholipid distribution between the inner and outer monolayers (the ***flip-flop*** mechanism).

Exposure of phosphatidylserine to the exterior surface of the membrane has been linked to the onset of the execution phase of apoptosis, experimentally supported by annexin-V binding to phosphatidylserine.

Phosphatidylserine transmembrane movement, as produced by the *flip-flop* mechanism, results in the uptake of the APOP*ercentage* Dye by apoptotic committed cells (Figure 3). Dye entry does not take place until the '*flip-flop*' mechanism has occurred. The APOP*ercentage* Dye enters the cell following this event and dye uptake continues until *blebbing* occurs. No further dye can then enter the now defunct cell and the dye that has accumulated within the cell is not released.

THE APOPTOTIC PROCESS

[1] A prolonged, sequence of internal and external checks and re-checks are performed before a cell is permitted to 'self-destruct', a TINA option. (TINA: **t**here **i**s **n**o **a**lternative).

[2] The 'melt down' of the cell is a self contained operation, where the interior components and structural elements are dismantled, within an enclosed and intact cell membrane.

[3] Apoptotic cell membranes retain the bilayer structure, composed mainly of phospholipids. This sealed unit prevents the release of cell components, some of which can provoke an inflammatory response.

[4] The key role of an intact cell membrane is again emphasised in the final stages of apoptosis, where the complete residual intercellular end-products are converted into small 'bite sized' packages (blebs), each complete with a bilipid membrane coat. These blebs are collected and ingested by the phagocytotic action of neighbouring cells.

[5] Apoptosis, therefore, appears as a *quiet* process to other cells and ensures that overall homeostasis is maintained.

[6] Apoptosis is an essentially normal physiological process, particularly during embryonic development and early growth to remove, now redundant, cells. In adult animals the process removes cells that are irreparable.

[7] The apoptotic process is also involved in many major diseases such as cancer, where transformed tumour cells have their apoptotic process disabled; permitting cell cycling to continue unchecked. In contrast some forms of senile dementia may result from excessive apoptotic induction of neural cells.

ASSAY PROTOCOL

ASSAY PREPARATIONS – options and suggestions.

Format: The APOP*ercentage*™ Assay has been designed for use with anchorage dependent mammalian cells seeded in sterile tissue culture containers. A 96-well multiwell plate format has been used as a working example of the assay, where wells can contain a concentration range of known or suspected apoptotic inducers. Other multiwell plates, or indeed microscope chamber slides, may be more suitable for other research experiments

Base Rate: Occurrence of apoptosis within a particular cell line should be assessed to examine the frequency of dye labelled cells. The incidence of apoptosis within a specific cell line will vary due to cell age, number of sub-cultures (cell passages) and culture conditions.

A healthy cell line seldom contains more than five dye labelled cells within a population sample of ~40,000 cells, as contained within one confluent well of a 96-well plate (see Figure 1[a]). Higher numbers of labelled cells may indicate a *'sick cell population'* (microbial/viral infection), or trace amounts of an unsuspected apoptotic contaminant (in the medium or serum).

Positive Control: A reproducible apoptotic compound should be considered to induce apoptosis within selected cell lines. The inducer compound can then be included in a few wells within each 'test' experiment, to act as a 'positive' apoptotic control (see Figure 1 [b]).

An effective apoptotic inducer, which has been found to work with many cell lines, is fresh 5mM hydrogen peroxide. It has the advantage of a short induction period; approximately two hours. Unlike many xenobiotic apoptotic agents it has low toxicity (to the researcher). This low cost agent readily dissolves in the culture medium. Treatment of most cell lines with 5mM hydrogen peroxide for four hours, should induce approximately 100% apoptosis; without too much cell loss (see Figure 1).

Cell Adhesion: The addition of experimental apoptotic agents to the culture medium can often have an unfavourable effect on cell adhesion, resulting in detachment and 'rounding-up'. Firm anchorage attachment of the cells, prior to adding the apoptotic test agent, can help to minimise this adverse effect.

Extensive trials, using coated wells (collagen, gelatin, polylysine) found that a 'deep layer' of a weak gel of gelatin was particularly effective in providing uniform cell growth and preventing some cell loss caused by apoptotic inducers. Other cell types may differ and test trials to ascertain suitable matrix composition is recommended. If unacceptable cell loss is encountered with your test agent(s), consider growing the cells in gelatin (see note on page 9).

CELL TREATMENT AND DYE LABELLING

[1] Due to considerable variations in the growth rate and the diverse range of mammalian cells' that are maintained *in vitro*, the following protocol should be considered as a 'start-up' guide. Before beginning this protocol please see the note below.

[2] Seed a 96-well tissue culture plate with between $2x10^4$ and $5x10^4$ cells/well, in 200µl appropriate culture medium.

[3] Incubate the cells at 37°C/5% CO_2, until confluence is reached (for most cell lines approximately 24 h).

[4] Remove the incubation culture medium and add 100 µl/well of the apoptotic inducer/inhibitor to be tested, in fresh culture medium. Incubate the apoptotic inducer/inhibitor for the appropriate amount of time (NOTE: The time taken to induce apoptosis will vary depending on the apoptotic inducer/inhibitor used – see pages 16-19 for examples).

[5] 30 min before the apoptotic inducer/inhibitor incubation time is reached, add 100µl/well fresh culture medium containing the apoptotic inducer/inhibitor and 5µl/well APOP*ercentage* Dye. (NOTE: It is advantageous to make a stock solution of APOP*ercentage* Dye and culture medium [containing the apoptotic inducer/ inhibitor] .

[6] Incubate with the APOP*ercentage* Dye for the remaining 30 min, at 37°C/5% CO_2.

[7] Syringe off the culture medium, apoptotic inducer/inhibitor and dye mixture. Gently wash the cells twice with 200µl/well PBS; to remove unbound dye. (NOTE: Be careful to pipette gently as the apoptotic process will loosen cells).

NOTE: Cell adhesion using gelatin (recommended)

The assay kit contains sterile 0.4% gelatin, for cell attachment to a gelatin matrix. Growing cells in the gelatin is highly recommended when quantifying the levels of apoptosis using the ADP* technique (see page 12), as it leads to uniform cell growth – and provides reproducible pixel count values. It also prevents a certain amount of cell loss caused by apoptotic agents; therefore increasing colorimetric, fluorimetric and pixel count values obtained using the assay.

[1] Add 100 µl/well 0.4% gelatin to a 96-well tissue culture plate.

[2] Seed cells at $2x10^4$ – $5x10^4$ cells/well, in 200 µl culture medium, on top of the gelatin.

[3] Incubate the plate at 37°C/5% CO_2 until confluence is reached.

[4] Remove the gelatin, culture medium solution and rinse cells once with 100µl of fresh medium.

[5] Carry out the APO*Percentage*™ Assay as detailed above from step 4 onward.

APOPTOSIS QUANTIFICATION

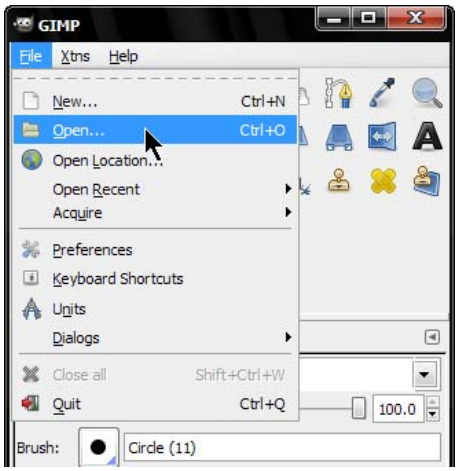
Analytical Digital Photomicroscopy

Digital images of APOPercentage Dye labelled cells can be used for apoptotic cell quantification. Using GIMP (or Adobe® Photoshop®), the level of apoptosis can be measured and expressed as a pixel number. The method is for GIMP, a free software available for download from www.gimp.org, which runs on Mac, Linux and Windows. A protocol for Adobe® Photoshop® is also available from our website.

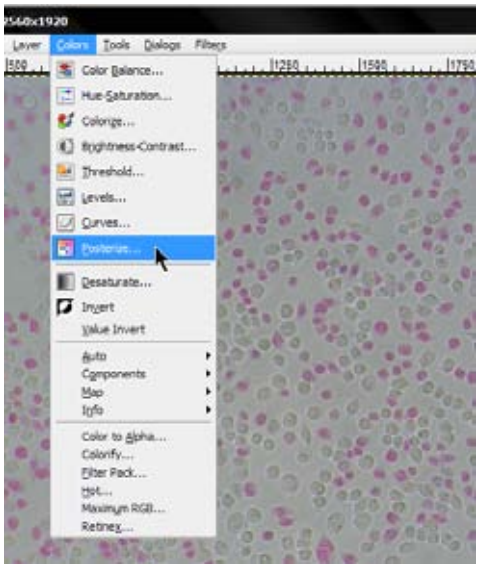
The white balance on the camera should be set using the control well and should give a pale gray background. The other settings on the microscope and camera such as iris aperture, brightness , picture quality, focus and zoom should remain constant within each experiment.

At least three photographs should be taken of each well to give an average pixel value. All photographs in an experiment should be at a constant magnification. The fields of view selected should contain around 200~400 cells and they should be of similar confluence to ensure pixel values are representative. This is especially important if smaller wells are used as cells tend to be more crowded near the edge of the well.

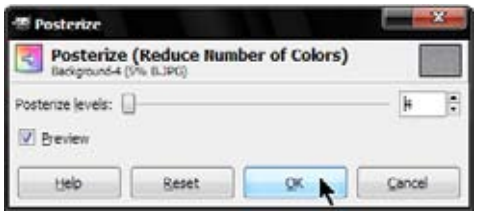
Once the images are on the computer follow the protocol outlined below to obtain pixel counts for each photograph. These values are then used to calculate the level of apoptosis between wells and can be expressed as a percentage of the control wells.



(1) Open the image to be analysed by clicking File → Open

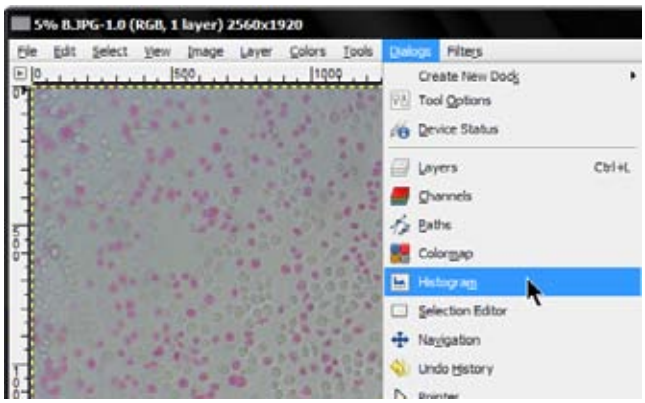


(2) Click Colors —> Posterize to prepare the image for pixel counting.

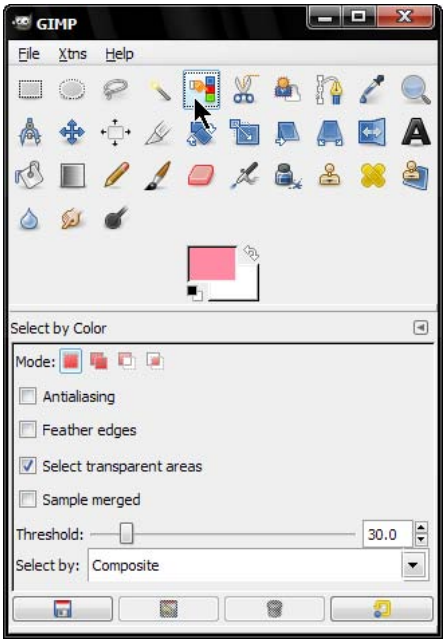


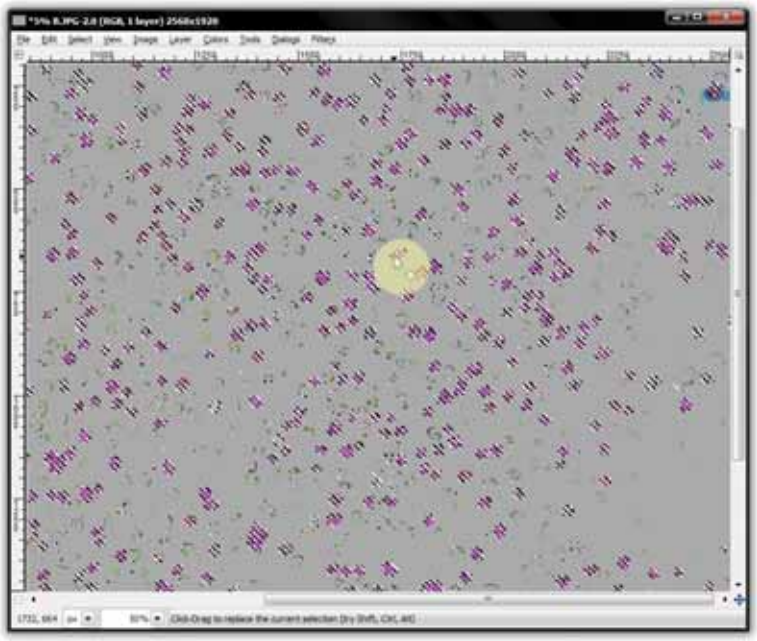
(3) Adjust the levels setting to the minimum number that turns stained cells into shapes of solid colour and the non-stained cells and background become uniform. (See Fig. 4) The minimum setting to use is 3, but 4 is ideal in most cases.

(4) Click Dialogs —> Histogram to open pixel counter

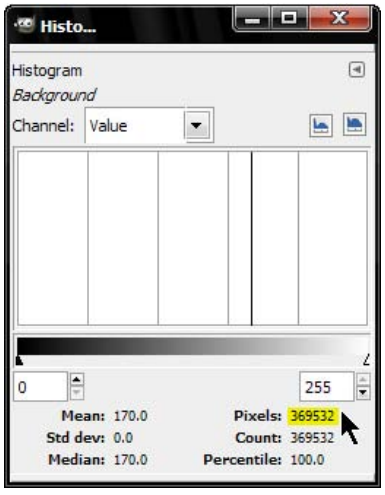


(5) Click the 'select by color' tool. Adjust settings so that they reflect what is shown in this diagram. It is important that these settings remain constant between the images being compared.





(6) Click on the solid colour of one of the cells to select all of them. The selection will be surrounded by 'marching ants' in the viewer window.



(7) Record pixel number from the histogram window.
Repeat these steps for each photomicrograph using the same posterization level to give comparative pixel numbers.

It is advisable to express apoptosis levels as a percentage of the positive control instead of as actual pixel numbers as this allows comparisons to be drawn between experiments.

NOTE: The ADP* technique described here may have applications for other digital photomicrographs you have. Biocolor has no copyright restrictions on use of the term ADP*. However, should you publish work using the ADP* technique, we would be grateful if you would cite this publication as your reference source.

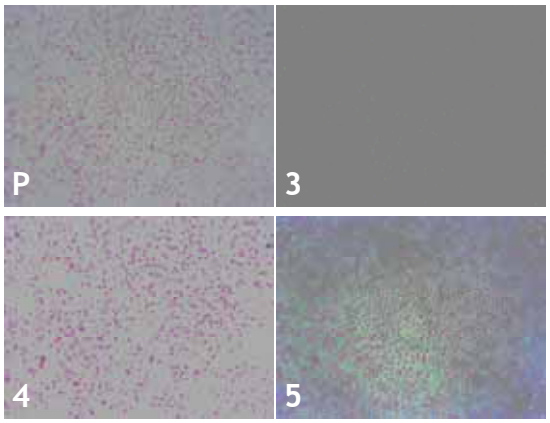


Fig. 4 Effects of Posterization on a Photomicrograph

Confluent CHO Cells were treated with 5% Ethanol for 1 hour and stained using APOPercentage Dye. A Photomicrograph was taken of the stained cells (P). It was then subjected to posterization at various levels in GIMP (indicated by numbers on figures).The fourth level of posterization produces the optimum image in this example.

Colorimetric and Fluorimetric Detection

Colorimetric or fluorimetric quantification are available but lack sensitivity, especially at low apoptotic levels.

After thoroughly washing the cells, add 100 µl/well APOP*ercentage* Dye release reagent and shake the plate gently for 10 min.

[a] Cell bound dye recovered into solution can then be measured using:

(i) a microplate colorimeter – absorbance measured at 550 nm

or

(ii)a microplate fluorimeter – fluorescence measured at excitation and emission wavelengths of 530 nm and 580 nm, respectively (with a cut-off of 570 nm).

[b] Both the absorbance and fluorescence measurements can be boosted by pooling 100 µl/well samples from 3 wells, to 300 µl in one well (see Figure 6).

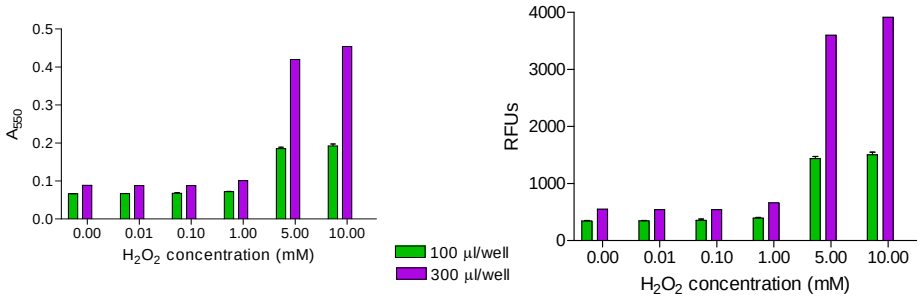


Fig. 6

The effect of sample volumes on absorbance and fluorescence values

5x10⁴ 3T3 cells were left to become confluent overnight, and then treated with 0 to 10mM hydrogen peroxide (H_2O_2) for 4 h. APOPercentage Dye was added to the cells as described in the Assay Protocol above.

Pooling the samples to 300 µl/well significantly enhanced A_{550} and RFU measurements.

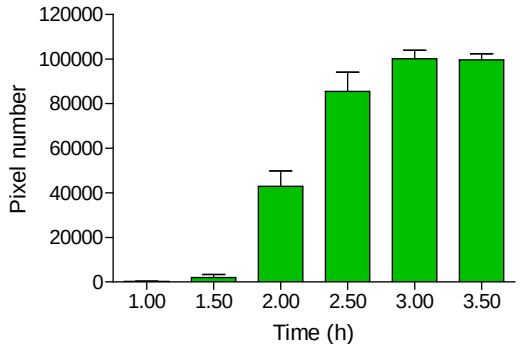


Fig. 7

Graph showing pixel number versus time, for treatment of 3T3 cells with 5mM H_2O_2

Confluent 3T3 cells were treated with 5mM H_2O_2, for the times indicated. After exposure to APOPercentage Dye, the apoptotic cells were quantified using the ADP technique, as described on page 10. The pixel number correlated with the visually observed level of apoptosis induced, and A_{550}/RFU measurements (see Figure 8).*

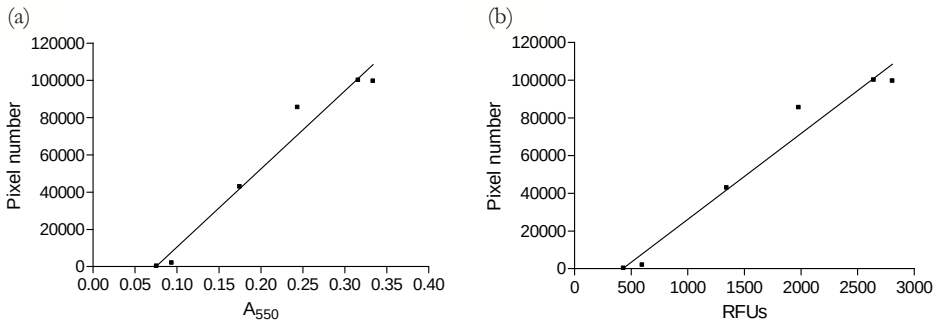


Fig. 8

Graphs to show the correlation of pixel number with (a) absorbance (A_{550}), and (b) fluorescence (RFUs)

The pixel numbers, obtained after the treatment of confluent 3T3 cells with 5mM H_2O_2 for 4 h (Figure 7), were correlated with A_{550} and RFU values from the same experiment.

There is a high level of correlation between the pixel number and A_{550}/RFU values ($r^2 = 0.971$). Thus, ADP is a viable technique for sensitive quantification of apoptotic cells labelled with APOPercentage Dye – and it makes a computer into an analytical instrument.*

[1] Hydrogen peroxide (H_2O_2)

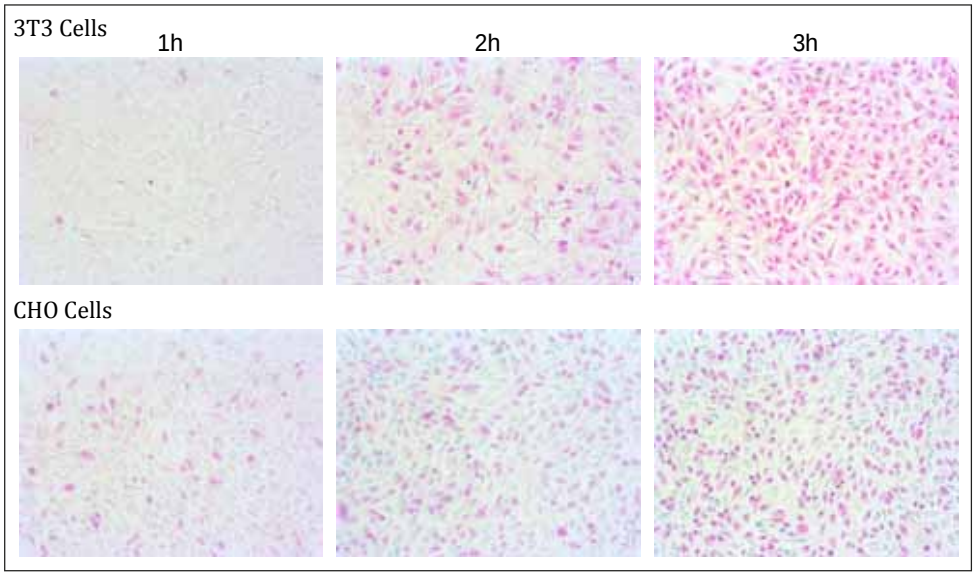


Fig. 9

Digital images of 3T3 and CHO cells treated with 5mM H_2O_2 for 1, 2 and 3 h

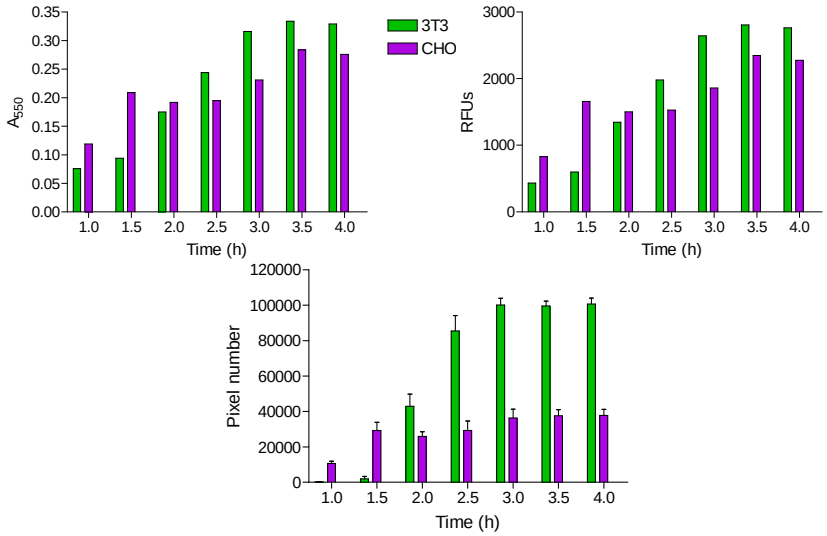


Fig. 10

Graphs showing the A_{550}, RFU (300 µl/well pooled samples) and pixel count values for 3T3 and CHO cells treated with 5mM H_2O_2 for up to 4 h.

[2] Cyclohexamide (CHX)

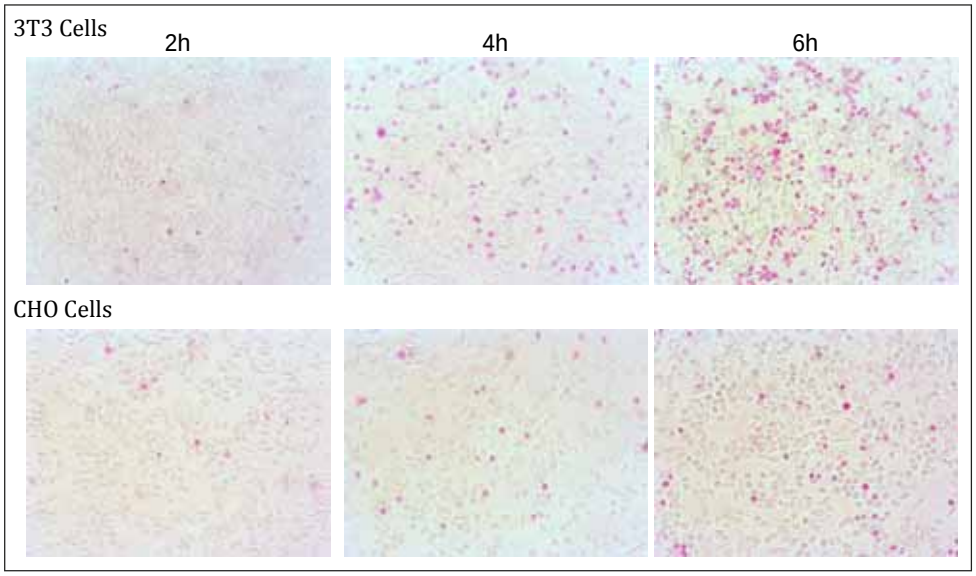


Fig. 11

Digital images of 3T3 and CHO cells treated with 10mM CHX for 2, 4 and 6 h

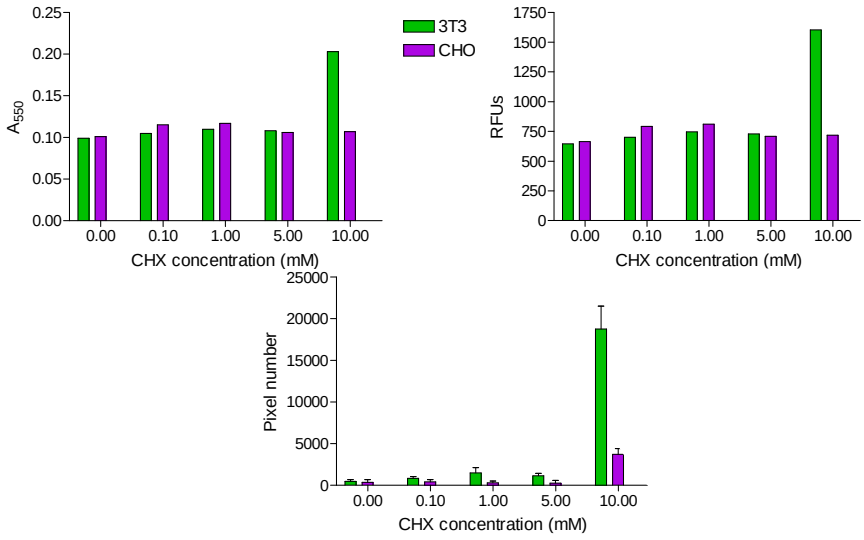


Fig. 12

Graphs showing the A_{550}, RFU (300 µl/well pooled samples) and pixel count values for 3T3 and CHO cells treated with up to 10mM CHX for up to 6 h.

[3] Cisplatin (CDDP)

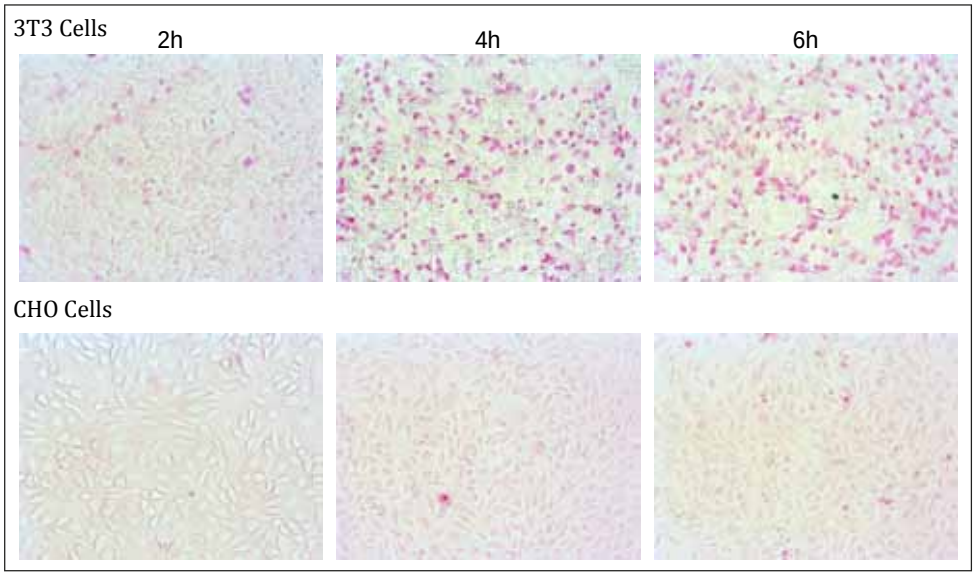


Fig. 13

Digital images of 3T3 and CHO cells treated with 2mM CDDP for 2, 4 and 6 h

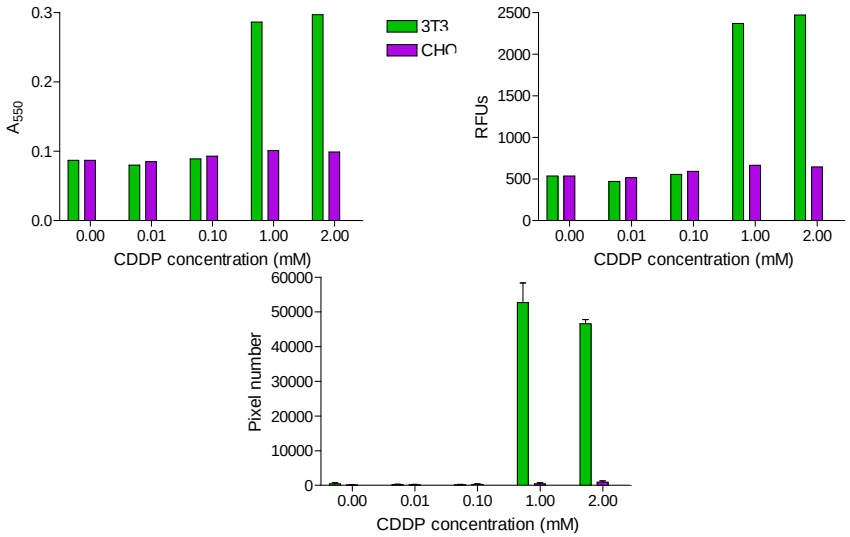


Fig. 14

Graphs showing the A_{550}, RFU (300 µl/well pooled samples) and pixel count values for 3T3 and CHO cells treated with up to 2mM CDDP for up to 6 h

[4] 5-Fluorouracil (5-FU)

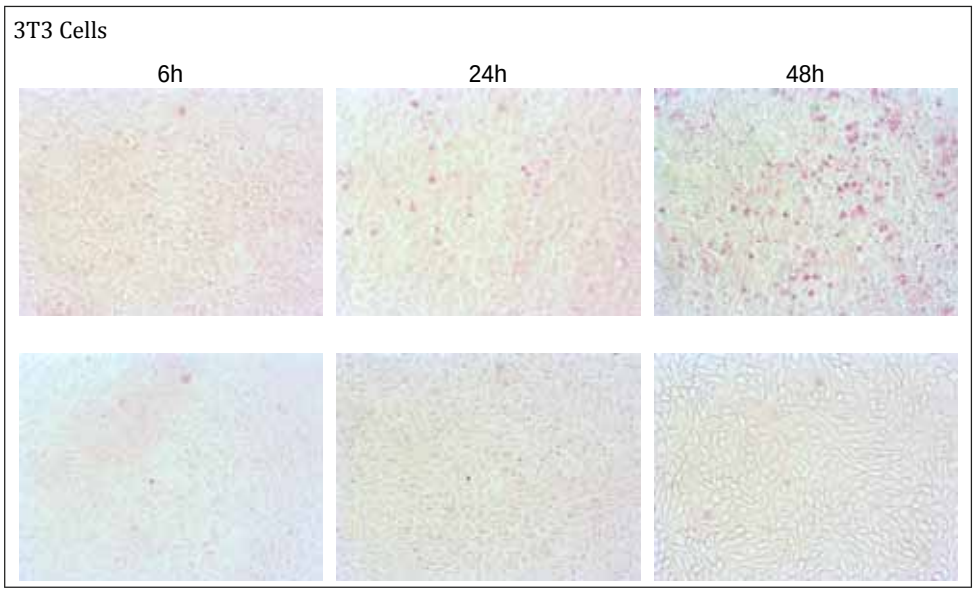


Fig. 15

Digital images of 3T3 and CHO cells treated with 10mM 5-FU for 6, 24 and 48 h

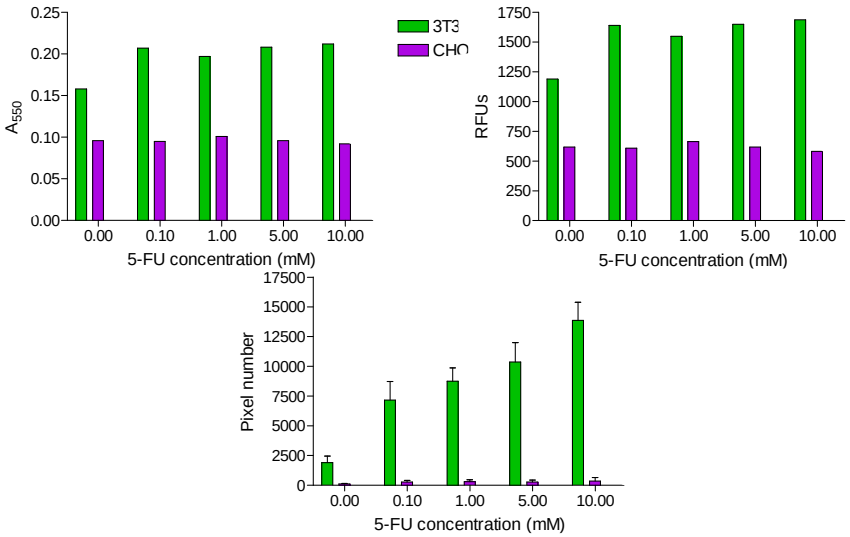


Fig. 16

Graphs showing the A_{550}, RFU (300 µl/well pooled samples) and pixel count values for 3T3 and CHO cells treated with up to 10mM 5-FU for up to 48 h

Apoptosis quantification:

Once unbound dye is washed off, the level of apoptosis may be quantified by:

[1] Analytical digital photomicroscopy and/or

[2] Colorimetric detection and/or

[3] Fluorimetric detection

See manual pages 10-13 for details on the above methods.

Record and graph data obtained using different quantification methods.

Analytical Digital Photomicroscopy (ADP) using GIMP

GIMP is cross-platform open-source software available for Mac OS X, Windows and many Unix distributions, such as Ubuntu or Free BSD.

It is regularly updated and is a viable and freely-available alternative to photo-image manipulation packages such as Adobe Photoshop®.

Depending on the host OS being used it can be downloaded from

- www.gimp.org/windows
- www.gimp.org/unix
- www.gimp.org/macintosh

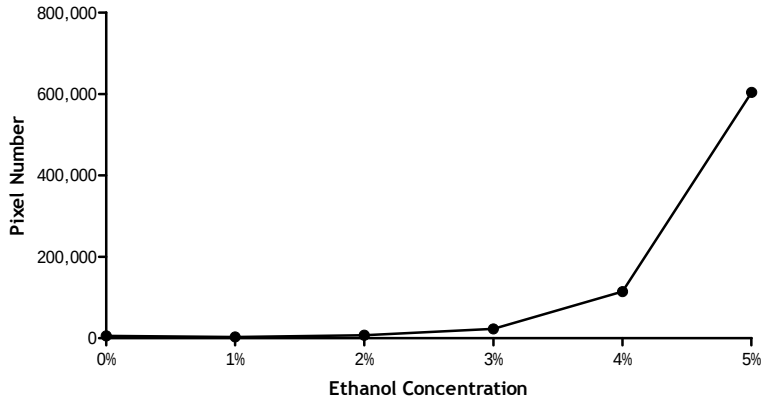


Fig. 3

Graph showing the pixel count values for CHO cells treated with up to 5% Ethanol for 1 hour demonstrating the 'TIPPING POINT' action of many apoptosis inducers

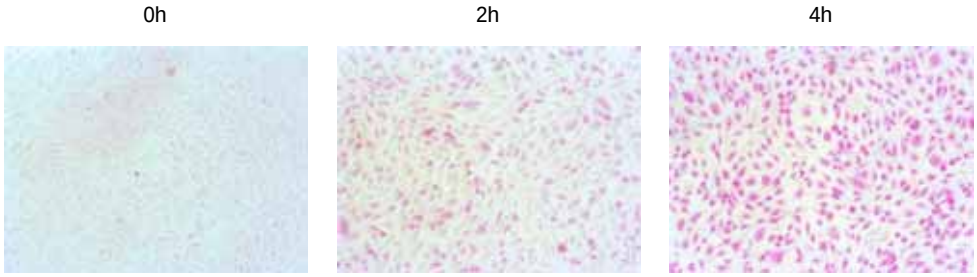


Fig. 1

digital images of CHO cells treated with 5mM hydrogen peroxide (H_2O_2) for the times above and then labelled with APOPercentage Dye

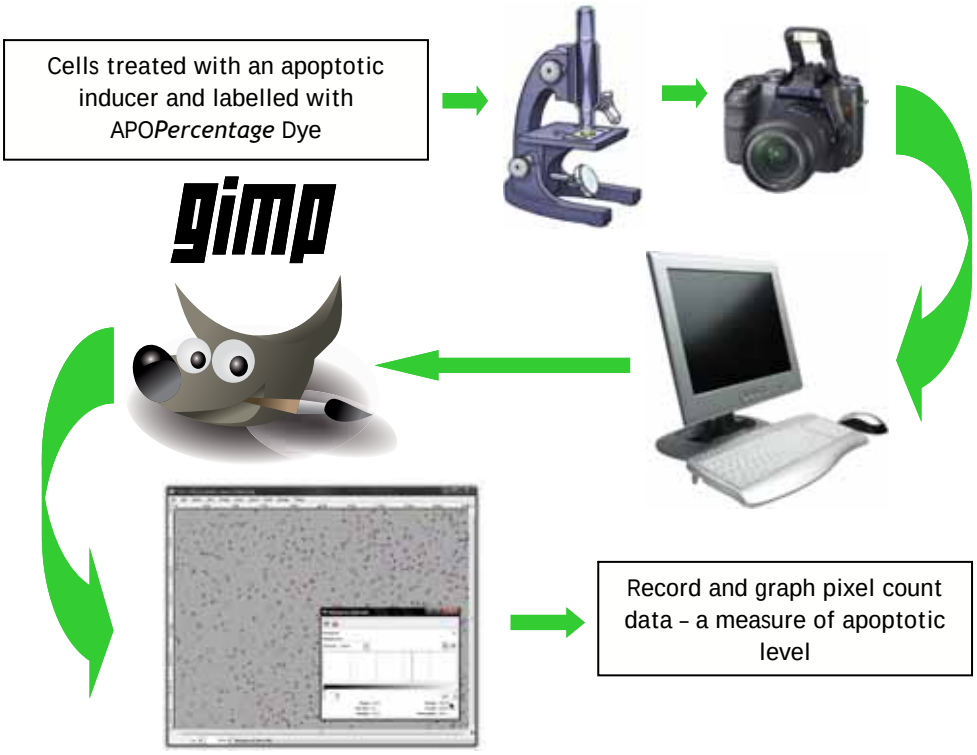


Fig. 2 Analytical Digital Photomicroscopy (ADP*)

Sensitive quantification of apoptotic levels in anchorage-dependent mammalian cells

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