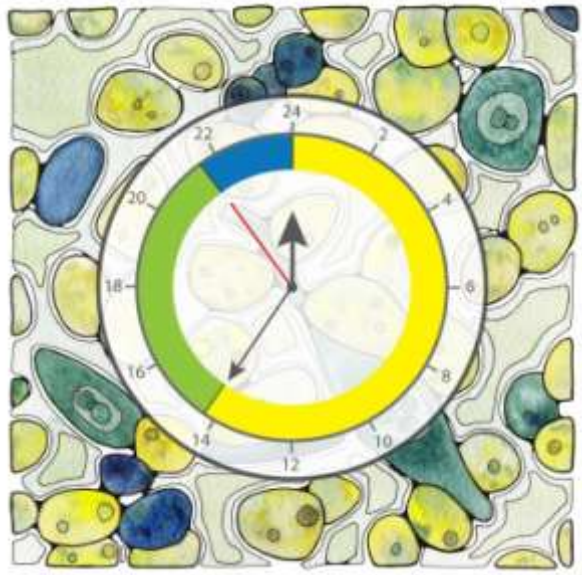
Cell-Clock

Cell Cycle

Assay

Detection and measurement of cell cycle phases



biocolor

life science assays

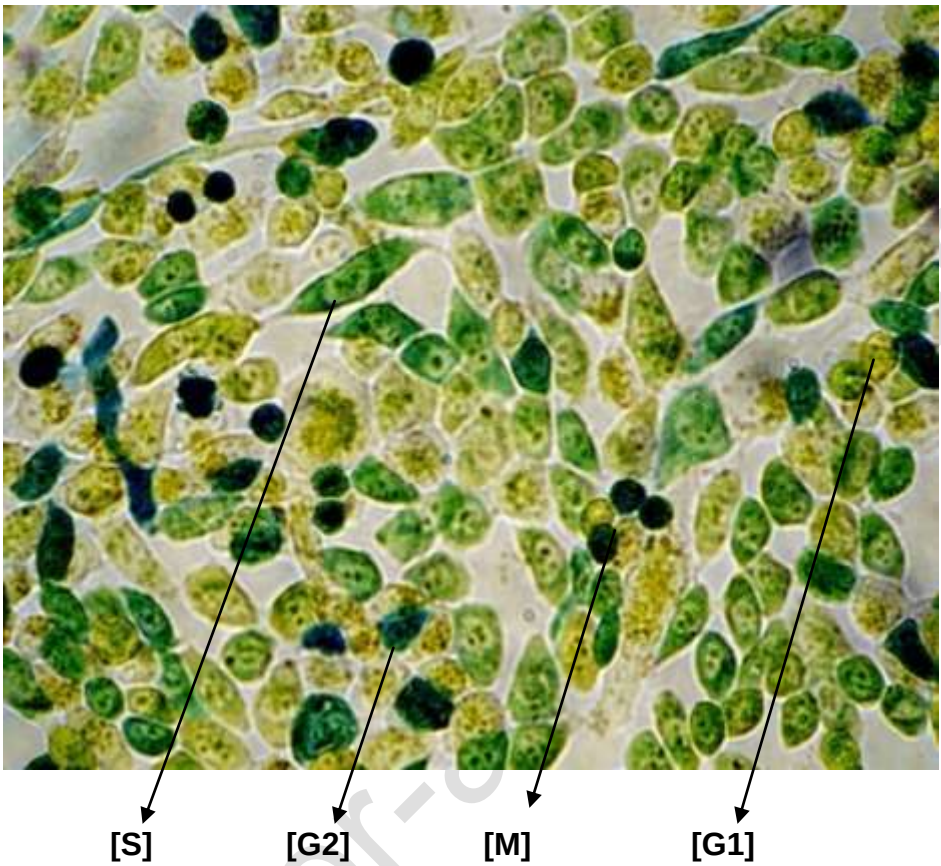


Fig. 1 Cell-Clock dye labelling of the four major phases of the cell cycle.

G1 cell: Increases in size from a small, pale yellow, newly formed cell, circular in shape. If exposed to a cycle arrest agent the G1 cell can become swollen in size.
S-phase cell: An example of a cell about mid-way through the S phase. Note the paler labelling of the nucleus.
G2-phase cell: During the G2 phase the cell becomes dark green in colour and will leave this phase as a blue cell.
M-phase cell: The cell has entered the final phase of the cycle. The cell becomes intensely blue and undergoes a distinct morphological change. A M-phase cell takes on a dumb-bell shape that will quickly transform into two spherical cells.In control wells, without any externally added arrest agents, the **Cell-Clock** dye labelled M phase cells eject the dye to reveal a dye free pair of newly formed daughter cells.
No toxic effects of the **Cell-Clock dye** have been observed, cycling time was unaffected**.**
Photomicrographs, (Fig.1-5), were obtained with live CHO cells, covered in a 2 - 4 mm layer of DMEM. Microscope magnification was x 100 or x 400.

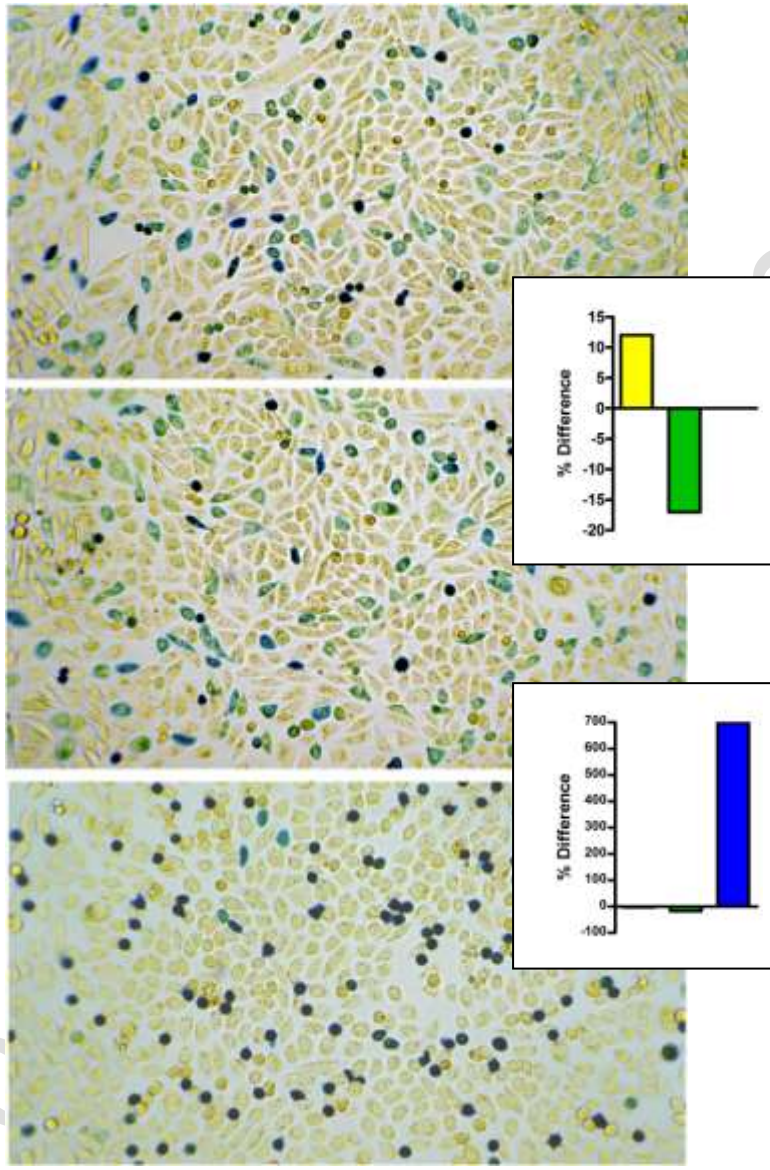
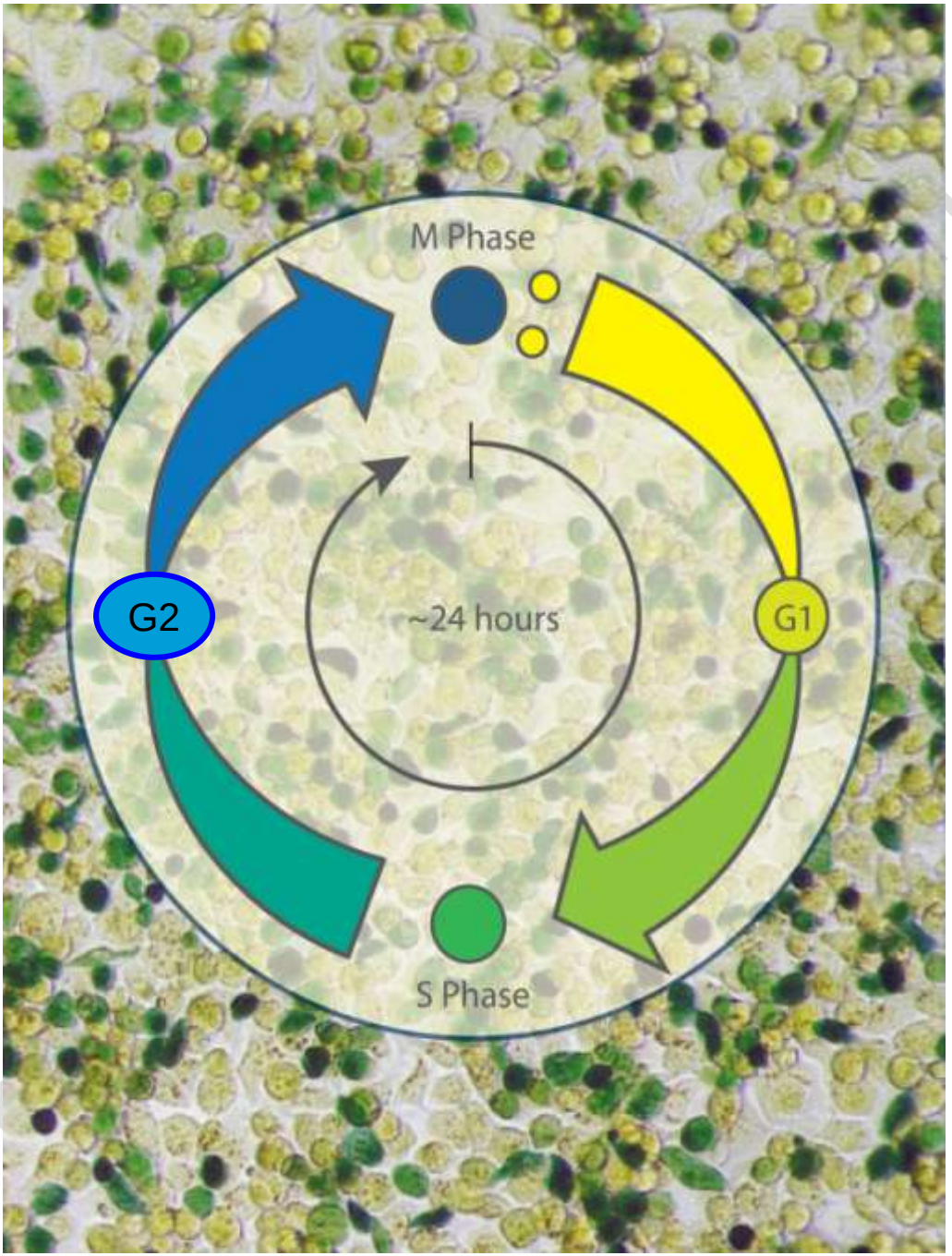


Fig. 5 M-phase arrest agent Docetaxel with cycling CHO cells.

Top image: Cells without exposure to external arrest agent(s)
Middle image Cells exposed to 0.25 uM DMSO for 5 hours.
Bottom image: Cells exposed to 1.0 uM Docetaxel and DMSO for 5 hours.
CHO cells from ECACC collection, (85050302). Microscope magnification x100.
Insert histogram-top: The percentage differences between Minus DMSO and Plus DMSO for blue, green and yellow percentage pixel values
Insert histogram-bottom:The percentage differences between Minus Docetaxel and Plus Docetaxel for blue, green and yellow percentage pixel values



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Cell-Clock™

Mammalian Cell Cycle

Assay

Detection and measurement of cell cycle phases

TECHNICAL INFORMATION

The Cell-Clock Assay has been designed for research work only.
Handle the Assay using Good Laboratory Practice.
Read the Manual before initial use of the assay.

www.biocolor-assays.com

ASSAY MANUAL

The **Cell-Clock** Assay is a live-cell detection and measurement system that can be employed to monitor the four major phases of the mammalian cell cycle during *in vitro* culture.

The assay uses a redox dye that is imported by live cells. Following dye uptake and incubation a distinct color change occurs within cells, with particular color changes being associated with cells in the G1, S, G2 and M phases of the cycle.

The ratio of cells within the different phases can be calculated using this assay and is supplemented by visual observation of the dye labelled cells in the photomicrographs.

The calculation of phase percentages is obtained by computer software analysis of the digitised images of photomicrographs using, **ImageJ** software.

The cell dye labelling time is one hour at 37^0C. The assay has been designed for use with 12 or 24 well cell culture plates, but can be adapted for other formats.

The dye reagent has three properties that make it suitable for use as a live cell bioassay.

[1] The reagent contains a *vital* dye that is taken up by *live* mammalian cells during *in-vitro* culture.

[2] The dye component of the reagent is a redox dye. In its reduced form the dye reagent is yellow in color, while in the intermediate state it is green before turning dark blue in the fully oxidised form.

[3] If mitosis occurs, the dye accumulated intracellularly is rapidly expelled prior to the formation of two pale yellow daughter cells.

Possible Assay Applications:

To monitor variations in cell cycling patterns caused by culture medium components, cell density, culture time or ageing associated with increasing sub-culturing, (cell passages).

To characterise cell cycle patterns that can be observed between different cell line collections.

To assess if a test compound, such as a potential or known anti-cancer drug, has an action / reaction within a specific cell cycle phase, (pages 9 & 11).

To monitor, analytically and visually, experiments to synchronise a colony of cells within a microwell using cycle phase arrest compounds or procedures, including how long before the test cell colony reverts to a mixed phase population.

Cell Cycle and Cell Redox Potential - a Comprehensive Review.

Redox Control of the Cell Cycle in Health and Disease.
Sarsour, E.H., Kumar, M.G., Chaudhuri, L., Kalen, A.L. & Goswami, P.C. **(2009)**
Antioxidants & Redox Signaling **11,** 2985-3011.

Cell-Clock Assay Pack Sizes and Storage Conditions.

Standard Assay Kit, Product Code: C1000, (100 assays in 24 well format)
Economy Assay Kit, Product Code: C2000, (400 assays in 24 well format)

Store in the dark, unopened at 4^0C; all components are sterile and stable for 6 months.
Once opened the assay components should be retained at 4^0C and used within 10 days.

Assay Kit Components

Cell-Clock Dye Reagent; 20 ml (C1000) or 20 ml x 4 (C2000)
The dye is prepared in Dulbecco's Modified Eagle's Medium, (DMEM).

Cell Dye Wash Reagent; 100 ml x 2. Dulbecco's Modified Eagle's Medium.

The above reagents should be stored at 4^0C and be pre-warmed to 37^0C before use.
Cloudy reagents, due to accidental microbial contamination must be discarded.

Cell culture plate, 1 x 24 microwell plate. Supplied in a sterile sealed pack.

Assay Manual
Further copies of this manual can be downloaded, free of charge, from our website.

Other consumables required. Further 12 or 24 well plates, (sterile).

Equipment required. Inverted-stage microscope with an attached digital camera, for subsequent image analysis of the photomicrographs.

A pre-installed copy of **ImageJ** is required for your computer**.**
This image processing software is available, free of charge, from **http://rsbweb.nih.gov/ij**

The ***ImageJ*** User Guide can also be down-loaded from:**http://imagej.nih.gov/ij/docs/guide** as a pdf file to provide a comprehensive guide.

It is available on screen using ***Adobe Reader*** or can be produced as a print document.

ASSAY PROTOCOL

DYE LABELLING OF LIVE CELLS

Assay users may need to make modifications to this protocol for a particular cell line or treatment regime.

[a] Seed a 24 well cell culture plate with between $2x10^4$ and $2x10^5$ cells / well in 500 µl of culture medium, with serum supplement if required.

[b] Incubate cells at 37^0C, 5% $C0_2$, to >80% confluence (overnight). Slow cycling cells may require either a higher seeding density or a longer incubation time.

[c] Remove spent culture media. Proceed to step **[d]** without delay.

[d] Add the experimental test compound in 500 µl of fresh culture medium to each well. Time and concentration trials are usually required to establish contact time for maximum effect of untested agents. Each plate should include wells of cells in a treatment-free medium as a control.

[e] Following incubation with agent(s) add 150 µl of **Cell-Clock** Dye Reagent to the centre of each well. Do not swirl or shake the plate. Return the plate to the incubator for a further 60 minutes.

[f] Remove the Dye Reagent and culture medium, gently wash cells with 500 µl per well of warm DMEM. (See Note below for protocol modification if cells become detached).

[g] Remove wash from wells and replace with 200 µl of fresh warm DMEM. Pipetting should be done gently with pipette set to 500µl when removing wash to avoid cell loss.

[h] A photographic record must now be obtained from each well within 15 minutes of the 200µl DMEM addition, before the cell color labeling pattern diffuses.

Note: If experimental treatment causes detachment of cells the following modification to step **(f)** of the protocol can be adopted. The **Cell-Clock** Dye Reagent plus spent medium (containing detached cells) is removed and centrifuged (2000 r.p.m. for 3 minutes) to recover the cell pellet. Most of the supernatant is carefully removed and the cell pellet then re-suspended in 125 µl of warm DMEM. Return without further delay to original wells, (or place in new wells). The cells, (attached ± detached cells), can now be photographed.

Assay Development: Test cells examined.

Most of the R&D work was carried out using CHO and 3T3 cell lines obtained from ECACC.

Other cell lines examined were V79, VERO, BHK and HT1080.

OBTAINING A PHOTOGRAPHIC RECORD

A digital camera attached inverted stage microscope with magnification x 100 is required.

Higher magnifications, (magn. x 400), can be used although this results in a reduction in the number of cells per image but provides better morphological details of the cells.

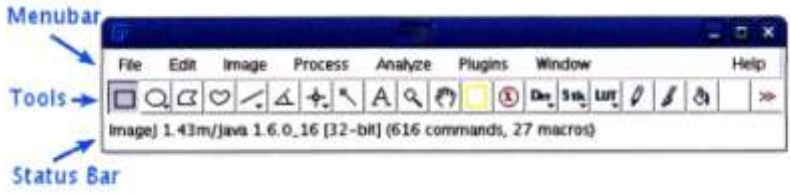
Capture 2 or 3 images per well, from areas of similar cell density to reduce errors caused by variations in cell distribution patterns within the wells of non-confluent cells.

Avoid the edge of wells as the cell density pattern can differ substantially from the flat surface of the rest of the well.

On completion of the photographic record transfer the digital images, (as jpg files), from the camera to a computer for storage and subsequent color pixel analysis.

CELL CYCLE PHASE QUANTIFICATION

[1] Open the ***ImageJ*** program; click **File > Open.**
Select and open the image to be analysed. The required image is loaded ready for analysis.



The image can be displayed at 50% of actual size by using the keyboard [**+**] key on the computer keyboard. Increasing the cell size image improves visual selection of cells without reducing the number of cells counted.

Color Pixel Selection:

[2] From ***ImageJ*** main menu window

Click **Image > Adjust > Threshold;**

In the Threshold window ensure that both brightness ***sliders*** are moved to the left and that **Default** and **Red** are selected.

Gradually move the ***bottom slider*** to the right until the dark blue cells are covered by red pixels, the slider values define the 'Threshold value range' used (see Fig. 2b). The computer processor counting of pixels can take several seconds to complete the selected colored pixel audit.

Retain a record of the **Threshold values** range.

Dark Blue Pixel Counting:

[3] From ***ImageJ*** main menu window

Click **Analyse > Analyse Particles**.

Set **Pixel^2** to **10-infinity**.

Tick '**Summarize'** and '**in situ show'** boxes. Click **O.K.**

The **Summary table** displays;
Count, Total Area, Av Size and Area fraction.
When the Summary is displayed it can be saved using **File > Save as**
Use the ***Total Area*** figures for Data Analysis,(page 7).

Green Pixel Counting:

[4] Return to the **Threshold** window to assay the green pixels.

Move the ***top slider*** to the same value as the ***bottom slider***, (to exclude the re-labeling of the dark blue cells).

Gradually move the ***bottom slider*** to the right until all dark green and medium green cells are displayed in red.

Click **Analyse > Analyse Particles** and the green pixel results are recorded in the open Summary table. Retain a note of the Threshold value selected.

Yellow Pixel Counting:

[5] Repeat the process by returning to the **Threshold** window to assay the yellow pixels.

Move the ***top slider*** to the same value as the ***bottom slider***, (lowers the threshold range to exclude the re-labeling of blue and green cells).

Gradually move the ***bottom slider*** to the right until yellow cells are displayed in red.

Click **Analyse > Analyse Particles** and record yellow pixel results.

Pixel Summary:

[6] Retain a record of the **Summary table** of the tricolor pixel counts using **File > Save** before going to the next image or if closing **ImageJ**.

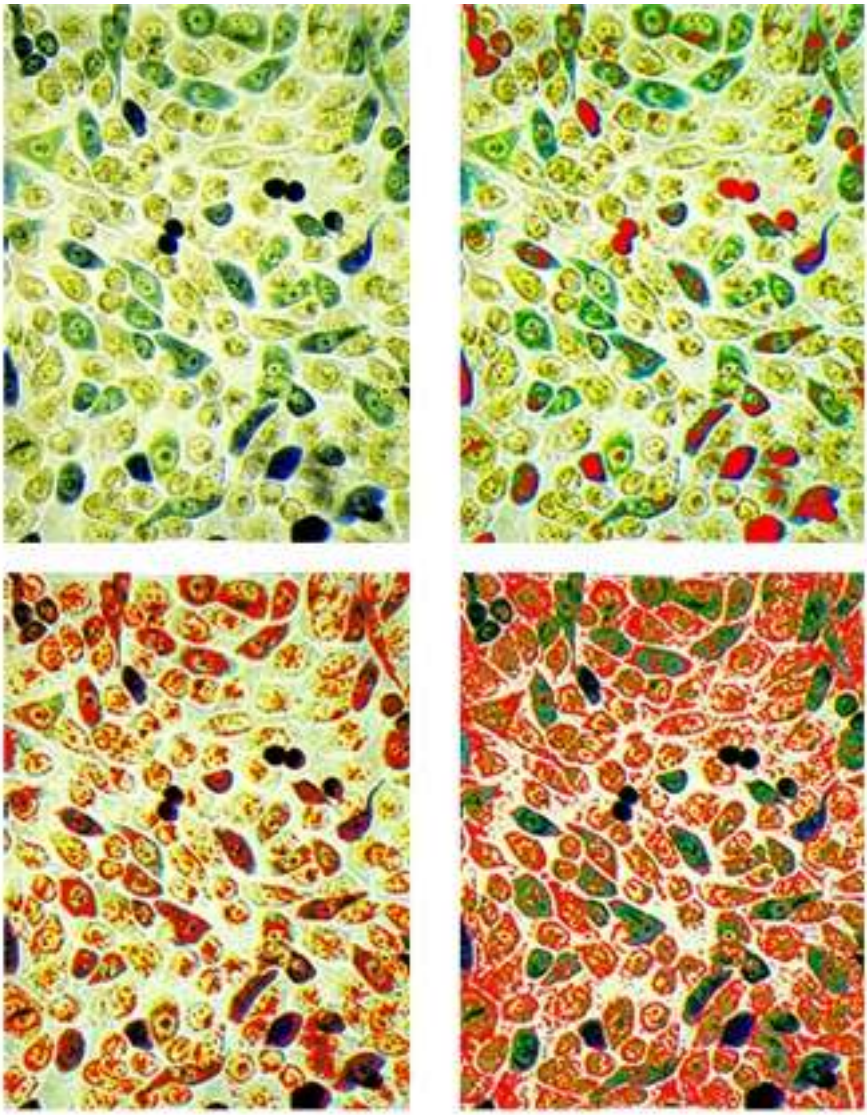


Fig. 2 Cell analysis of pixel color detection and counting using *ImageJ*

*top left image; Color of CHO cells after labeling with **Cell-Clock** dye reagent.*
*top right; Dark blue labeled cells selected by **ImageJ** displayed as red pixels.*
bottom left; Green cells selected and displayed as red pixels.
bottom right; Yellow cells selected and displayed as red pixels red.

DATA ANALYSIS:

CALCULATION OF PERCENTAGE OF BLUE, GREEN AND YELLOW PIXELS

Add the number of blue, green and yellow pixels obtained from the photomicrograph to obtain the Total Cell Colored Area, (in pixels).

Test example:

An analysis using CHO cells cultured in DMEM/F12 medium containing 5% calf serum supplement; culture time 22 hours, (Fig.2).

Express the blue, green and yellow pixel counts as percentages of the total colored pixels.

Yellow pixels represent the **GO/G1** phase cells.

Green pixels represent the **S** phase cells.

Dark blue pixels represent late **G2** and **M** phase cells.

Dye Colored Cells	ImageJ threshold red slider value	Number of pixels labeled red	Percentage of pixels labeled red
Dark Blue	75	452,052	6%
Green	170	2,095,921	28%
Yellow	230	4,982,241	66%
Total Colored Cells		7,530,214	100%

Further examination is based on the above pixel analysis process and involves comparisons of the percentage differences for the percentage of blue, green and yellow cells between controls and test samples;

$$\frac{\text{Test \% blue} - \text{Control \% blue}}{\text{Control \% blue}} \times 100 = \text{Blue pixel \% Difference.}$$

The same formula format is also used for green and yellow pixels percentages, (see examples on Pages 9 to 11).

CELL PHASE PATTERNS DURING EXPONENTIAL GROWTH AND AT CONFLUENCE

There is a significant difference in cell shapes and cell colors of cell populations when examined during exponential growth and the same cells when sampled at confluence, (Fig 3).

During exponential growth the S-phase of the cycle is the 'rate controlling step' whereas at confluence the cell proliferation is restricted by the lack of cell free matrix space until M-phase cells are released from the matrix.

The dominance of yellow-green cells observed during exponential growth contrasts with the gradual accumulation of dark blue M-phase cells when the cell sample within the well becomes confluent, (more than 80% of the surface covered by cells).

Cultured for 24 hours **Cultured for 48 hours**

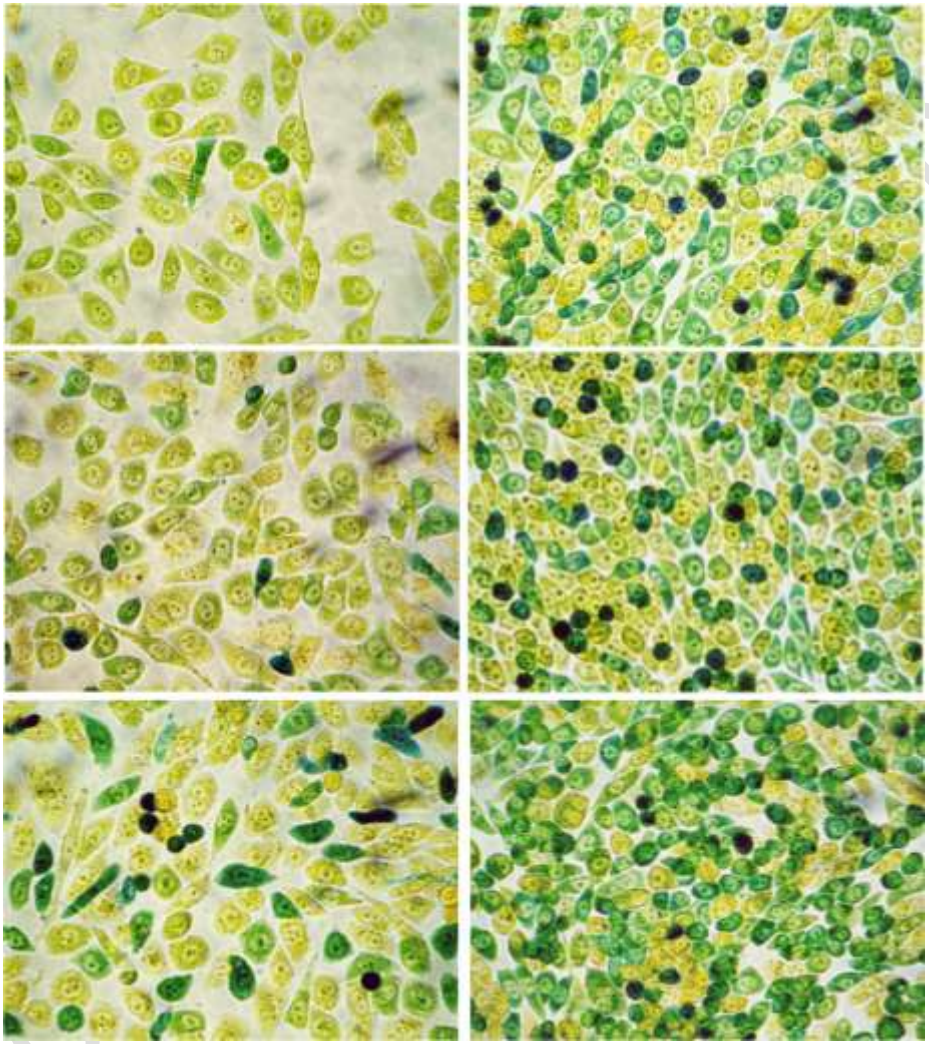


Fig. 3 Cell phase patterns during exponential growth and at confluence.

Effects of seeding density and culture time of cell line CHO

Top row - low cell density, 5 x 10^4/well using a 24 well plate

Middle row - medium cell density, 1 x 10^5/24 well plate

Bottom row - high cell density, 2.5 x10^5/24 well plate

Left column - cells cultured for 24 hours. Right column - cells cultured for 48hours.

Assay Applications

[1] Early S-phase arrest to obtain cell cycle phase synchronization

The quest for a non-toxic procedure to convert asynchronous cell cycle phase cells to phase synchronized cells has generated much research;
(Mammalian cell cycle phase published papers; 534,000, **(2013)** *Google Scholar*).

Synchronization can be temporally achieved yet after a few hours from the removal of the arrest agent the cell population gradually becomes phase asynchronous. This may reflect the time interval individual cells have been held at the arrest step. The *in-vitro* cell cycle process is analogous to a city marathon where several thousand runners start together yet with time groups of runners become stretched out over the designated course stages.

A commonly used G1/S arrest agent is hydroxyurea, (Sinclair, W.K. **(1965)** *Science*, **150**, 1729-1731). Other G1/S arrest xenobiotic agents include aminopterin and cytosine arabinoside. Nutritional deprivation has also been employed such as reduction of serum content in the culture medium, (Merrill, G.F. **(1998)** *Methods in Cell Biology*, **57**, 229-249).

Test example: A mammalian anchorage-dependant cell line was grown in T-flasks until contact inhibition induced quiescence. The cell sample was then trypsinised and seeded into a 24 well cell culture plate at a concentration of 7.5×10^4 cells / well. The cells, in 500µl/well of culture medium, were initially left for one hour in the laminar-flow cabinet at room temperature to settle and attach to the plastic matrix before transfer to an incubator, (37^0C /5% CO_2).

A double hydroxyurea block was employed similar to the procedure described by Lin, W. & Arthur, G. (**2007**) *Internat. J .Biochem. & Cell Biol*., **39**, 597-605. Cells were incubated in DMEM/F12 medium with a 5% serum supplement at 37^0C, 5% CO_2 in either culture medium (minus HU) or in medium supplemented with 50 µM hydroxyurea, (plus HU) for 18 hours. All wells were then drained and washed in DMEM before undergoing a 24 hour recovery period in the above medium without HU. The medium was then replaced with fresh medium, again with and without HU as in the initial incubation and the cells cultured for a further 24 hours. **Cell-Clock** Dye Reagent, 150µl/well was added during the final hour of incubation. Dye labeling, washing and pixel labeling and counting were as described in pages 3 to 7 of this manual.

The hydroxyurea treatment resulted in an increase in the number of yellow and green cells in the G1/S phases of the cycle, few blue cells were found. Cells exposed to HU displayed some swelling in size but otherwise appeared to have normal morphology when compared with the HU free control, (Fig.4).

In many research reports of cycle arrest agents there is an absence of photomicrograph illustrations to examine cell morphology before and during experimental treatment. The **Cell-Clock** Assay provides a suitable histochemical style labelling system, using an inverted visible light microscope providing time selective phase dye labeling patterns.
Caution: Response variability to cell cycle arrest agents for different cell lines has been reported, (Blajeski, A. *et.al*. **(2002)** *J.Clin.Invest*. **110**, 91-99).

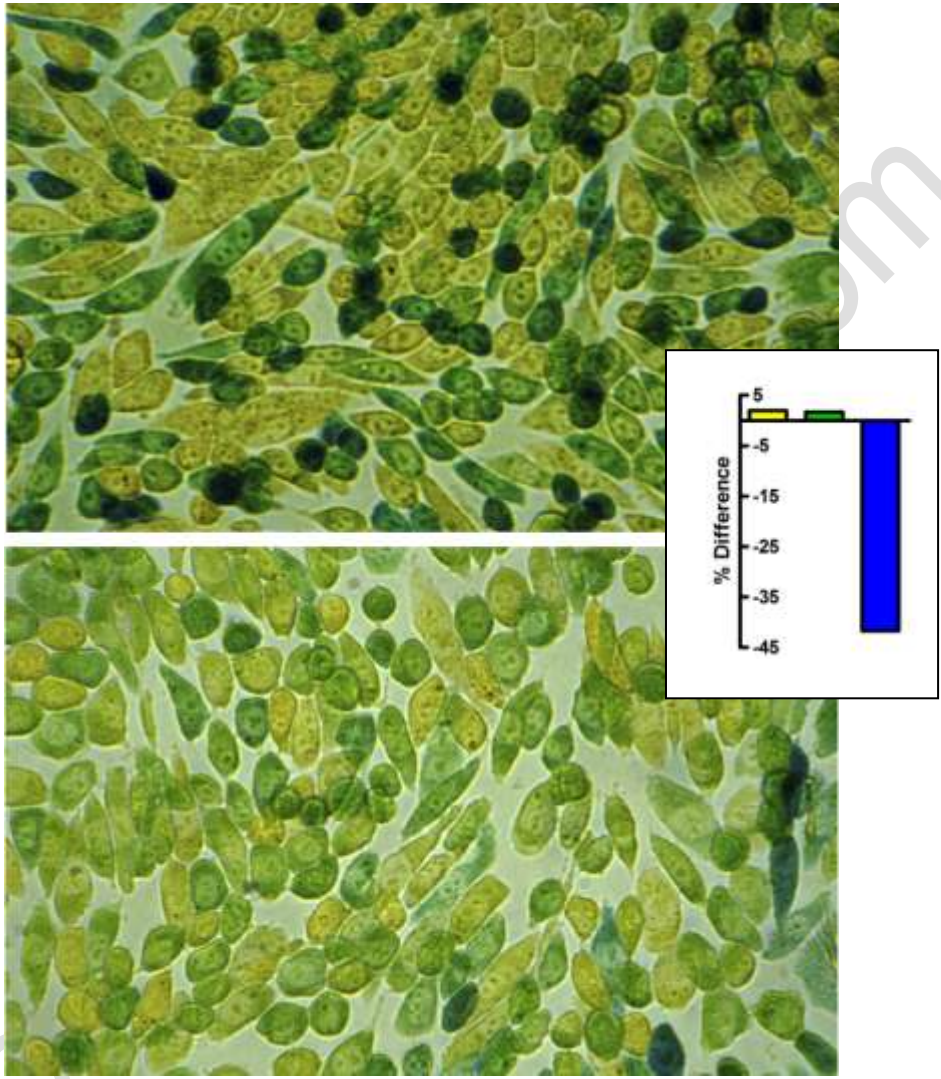


Fig. 4 Cell cycle S-phase delay following exposure to hydroxyurea.

Top image; CHO cells cultured for 67 hours, without hydroxyurea, (Minus HU).
Bottom image; same CHO cells/67 hours, including 50µM HU for 42 hours, (Plus HU).
Seeding concentration; 7.5 x 10^4 cells/well in a 24 well microwell plate.

Insert histogram: The percentage difference between Minus HU and Plus HU of the blue, green and yellow percentage pixel values.

Assay Applications:

[2] M-phase arrest analysis

The transition of a cycling cell into the M-phase stage is associated with a distinct morphological 'rounding-up' of the cell body, in preparation for mitosis. Within the cell, numerous cytoskeleton arrangements take place to facilitate chromosome segregation. This process can be inhibited using cell permeable anti-mitotic agents such as *Nocodazole, Taxol (docetaxel), or Vinblastine,* Tishler, R.B, Lamppu, D.M, Park, S & Price, B.D. **(1995**) *Cancer Research*, **55**, 6021-6025.

Test example: Cells were prepared and treated in a similar procedure to early S-phase arrest, (page 9), however instead of hydroxyurea an anti-mitotic compound was used; **Docetaxel.**

Cells were cultured overnight in 24 well cell culture plates before being treated with 1.0 µM Docetaxel with the aim to generate a cell population enriched with M-phase cells.
The **Cell-Clock** Dye reagent was added following exposure to Docetaxel for 5 hours and the assay as described on page 3 completed. Digital photomicrographs were obtained, (Fig. 5).

A series of trials are usually required to optimize the concentration / time for specific arrest agents required by different cell lines.

This test run was to establish if the dimethyl sulfoxide, (DMSO), used for the solubilisation of the solid Docetaxel was also a cell-phase inhibitor following dilution in DMEM to 0.002% DMSO. Higher concentrations, (>1.0%), of DMSO have been reported to cause G1 phase arrest, (Fiore, M, Zanier, R & Degrassi,F.(**2002**) *Mutagenesis*, **17**, 419-424).
A positive effect of M-phase arrest by Docetaxel was obtained. A small G1/S phase effect was found at low DMSO levels that does not require a replacement of this initial solvent for Docetaxel.

Assay limitations

[1] Photo-images of cells: Under optimal conditions a uniform lawn of cells covering the bottom surface of a well reduces the variation found between photo-images within individual wells that contain confluent monolayer cells.

More careful selection of photo-images is required for cells examined during exponential growth as these pre-confluent cell colonies vary in surface area size and are surrounded with gaps of cell free plastic matrix. To reduce this variation select three of the larger cell colonies for photo-imaging to permit an average value for pixel counting process.

Avoid selecting images at the edge of wells as cells in this location are atypical of the growth rate of cells in the flat base of the well.

[2] Pixel analysis of photo-images, (*ImageJ*). Although pixel counting is computer controlled the selection of the blue, green and yellow pixel ranges are under the control of the analyst through the pixel selection Threshold settings, (see page 5).

The analyst visually finds the Threshold range. Expressions of absolute pixel counts however are not recommended due to the variations between cell population images. Tri-color pixel counts are safer by comparisons when made between the blue percentage values of control wells and test wells, with the same system applied to yellow and green pixel percentages.
Pixel color data analysis of a microwell plate should be carried out by the same person, and if possible in a single session. A second opinion by a 'third party' can be obtained at any future time as the digital photo-images are stored on a hard drive or USB flash RAM.