

# At-line monitoring of cell cultures: Rapid cytometric evaluation of cellular physiology



## APPLICATION NOTE

**ABSTRACT** Mammalian cell culture processes face numerous challenges related to compressed product development cycles, capacity shortages, and the proliferation of cell lines and culture conditions. Underlying these efforts is the need for rapid, reliable techniques for monitoring cell viability at every stage of development, scale-up, and manufacturing. Conventional offline techniques for monitoring culture conditions are time- and labor-intensive, and incompatible with modern development and manufacturing timelines.

The Guava Personal Cell Analysis (PCA™) system simplifies complex cellular assays and shortens analysis times. Through its use of microliter-sized samples, the Guava PCA system reduces sample loss, and waste generation and handling. Guava PCA assays include automated analysis of cell number and viability, detection of protein expression, cell cycle analysis and apoptosis screening

In this note, we describe our studies comparing assays for cell number, viability, and apoptosis run on the Guava PCA system with standard microscopy methods. We assayed multiple cell lines of interest in bioprocess development and manufacturing. Cell count and viability results obtained using Guava® ViaCount® were compared with results from manual hemacytometer counting using trypan blue exclusion to assess cell viability. We compared apoptosis assay results obtained using the Guava Nexin™ application with results from manual counts of acridine orange and propidium iodide stained cells over a time course of apoptotic induction. The Guava PCA system yielded comparable results to standard methods with a high degree of reproducibility between experiments and operators. Large numbers of cells could be analyzed rapidly using the Guava PCA, producing data with higher statistical validity.



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## Guava Technologies

# At-line monitoring of cell cultures: Rapid cytometric evaluation of cellular physiology

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## Abstract

Mammalian cell culture processes face numerous challenges related to compressed product development cycles, capacity shortages, and the proliferation of cell lines and culture conditions. Underlying these efforts is the need for rapid, reliable techniques for monitoring cell viability at every stage of development, scale-up, and manufacturing. Conventional offline techniques for monitoring culture conditions are time- and labor-intensive, and incompatible with modern development and manufacturing timelines.

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In this note, we describe our studies comparing assays for cell number, viability, and apoptosis run on the Guava PCA system with standard microscopy methods. We assayed multiple cell lines of interest in bioprocess development and manufacturing. Cell count and viability results obtained using Guava® ViaCount® were compared with results from manual hemacytometer counting using trypan blue exclusion to assess cell viability. We compared apoptosis assay results obtained using the Guava Nexin™ application with results from manual counts of acridine orange and propidium iodide stained cells over a time course of apoptotic induction. The Guava PCA system yielded comparable results to standard methods with a high degree of reproducibility between experi-

ments and operators. Large numbers of cells could be analyzed rapidly using the Guava PCA, producing data with higher statistical validity.

## Introduction

The primary research interest of Prof. Mohamed Al-Rubeai's laboratory is animal cell technology; specifically cell culture optimization for growth and production of bioproducts, tissue engineering, and apoptosis. Among his current projects in the Department of Chemical Engineering at the University of Birmingham are medium optimization, regulation of cell cycle progression, and studies in cellular aging, gene therapy, and mathematical bioprocess modeling.

## Rationale for Rapid, Reliable Cell Testing

Cell culture productivity, a function of cell viability, is of particular concern at all stages of a cell culture process. In some situations cell viability may determine the quality of the final product. Apoptosis ("programmed cell death") may sometimes be delayed, and a culture's productive life extended, by adjusting media or other parameters.<sup>1</sup> Such rescue techniques, however, are only as effective as cell monitoring methods.

To quantify culture viability, scientists currently turn to flow cytometry or microscopic analysis.<sup>2,3</sup> Flow cytometry is expensive, requires highly trained operators, and consumes millions of cells per test sample. Most researchers rely upon the schedule of a core facility for conventional flow cytometry, making it difficult to perform time course experiments on live samples over periods of hours to days. Microscopy is

limited by inter-user variability, laborious and often time-consuming data collection, user fatigue, and small sampling size (i.e. a few hundred cells). Our group was interested in the potential benefits of replacing standard cell counting methods with the benchtop Guava PCA system, which promises ease-of-use, faster processing, less operator subjectivity, lower cell consumption per assay, and less waste. We evaluated the Guava PCA instrument and two Guava applications: Guava ViaCount (for absolute cell count and viability) and Guava Nexin (for apoptosis).

### Guava ViaCount Assay

Guava ViaCount provides absolute count and viability data on a variety of cell lines. Cells in suspension are stained using the Guava ViaCount reagent, which contains two DNA-binding dyes with different cell permeability characteristics. In a ViaCount assay the Guava PCA instrument draws cells into a capillary flow cell at a precisely controlled rate. Absolute cell counts are thus calculated from directly measured sampling volumes. The Guava PCA system differentiates viable from non-viable cells by detecting fluorescent signals from the DNA-binding dyes: one membrane-permeable dye stains all nucleated cells, while the second dye penetrates only cells with compromised membrane integrity, i.e. non-viable cells. The Guava PCA system only counts events associated with nucleated cell fluorescence in which forward light scatter (FSC) intensity is appropriate for a cell-sized particle. Debris events with low FSC are not counted. Live cells yield one fluorescent signal from the nucleated cell stain, and non-viable cells yield two fluorescent signals, from both DNA-binding dyes.

### Guava Nexin Assay

Apoptosis or programmed cell death is characterized by numerous alterations in the biochemical and physiological state of cells. An early event in apoptosis is the translocation of phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the cell surface. Annexin V has a high binding affinity for PS and thus readily binds to externalized PS, allowing detection of early apoptosis.

The Guava Nexin assay utilizes phycoerythrin (PE)-conjugated Annexin V and the cell-impermeant

fluorochrome, 7-aminoactinomycin D (7-AAD), to detect early apoptosis and plasma membrane integrity. Identification of three populations is thus possible:

- Viable cells: Annexin V(-)/ 7-AAD(-)
- Early apoptotic cells: Annexin V(+)/ 7-AAD(-)
- Late apoptotic and dead cells: Annexin V(+)/ 7-AAD(+)

Our laboratory was interested in assays that could be performed relatively quickly and simply, to allow monitoring or detection of a culture's response in real time. In work presented here, we monitored the changes in cell culture density and viability over time under different culture conditions using Guava ViaCount and manual hemacytometer counts with trypan blue exclusion. We also compared the results of several apoptosis time course experiments, comparing the Guava Nexin assay with manual counts using acridine orange/propidium iodide staining. We measured the change in culture viability after apoptotic induction with five agents over the course of many hours.

## Materials and Methods

### Cell line and culture conditions:

Recombinant Chinese hamster ovary cells (rCHO) and NSO wild type (NSOwt) mouse myeloma cells were grown under identical conditions in static batch culture in vented T75 flasks, 37°C, in a 95% air – 5% CO<sub>2</sub> humidified incubator, using DMEM/F12 media (Sigma, UK) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Austria). CHO320 cells, a recombinant CHO cell line producing human interferon- $\gamma$ , were grown in a 200-ml suspension batch using RPMI 1640 media (Sigma) supplemented with 5% FCS and methotrexate hydrate (MTX, 100  $\mu$ M).

Because rCHO cells are prone to aggregation, all cells were treated with Cell Disassociation Solution (Sigma) at 37°C. After 2 minutes cells were treated with serum-containing medium, centrifuged (167  $\times$  g, 5 minutes), and resuspended in the spent culture medium in which they had been cultured. Cells were analyzed immediately.

## Cell Count and Viability Assessments

**Manual cell count:** Recombinant CHO and NSOwt cell suspensions were prepared. For each sample, a 100  $\mu$ L aliquot of cell suspension was mixed with 100  $\mu$ L trypan blue (Sigma; 0.5% w/v in water) in microtiter wells (1:1 mixture). Stained cells were transferred onto the Neubauer haemocytometer and viewed under a phase contrast microscope at a magnification of 200x. Cells that excluded trypan blue were scored as viable, while those that stained blue (i.e. had lost membrane integrity) were considered non-viable. Cells from the top three and bottom three squares were counted on two separate grids.<sup>3,4</sup>

**Guava ViaCount Assay:** Stained rCHO and NSOwt cell samples were prepared by mixing cell suspensions with Guava ViaCount reagent at a 1:20 dilution (typically, 20  $\mu$ L of cell suspension with 380  $\mu$ L ViaCount reagent). After a 5 minute incubation, data were acquired from the stained samples using the Guava PCA system. Unstained cell samples diluted in dilution buffer were used to set instrument thresholds.

**Apoptosis Induction:** NSOwt in mid-exponential growth were inoculated into vented T25 flasks, allowing for a flask to be sacrificed at each time point over the 15-hour duration of the experiment. Flasks were incubated overnight (37°C, 95% air - 5% CO<sub>2</sub> humidified incubator) in DMEM/F12 media supplemented with FCS (10%). Apoptosis inducers were added to final concentrations listed in Table 1. Cells were sampled at 5, 10 and 15 hours after addition of inducer. Control cultures were not treated with an inducer.

**TABLE 1.** Inducers and concentrations used in apoptosis experiments

Inducer	Concentration
Staurosporine	4 $\mu$ M
Camptothecin	15 $\mu$ M
Farnesol	60 $\mu$ M
Etoposide	42.5 mM
Geranylgeraniol	50 $\mu$ M

*(all from Sigma Aldrich)*

**Manual Apoptosis assessment:** A dual staining solution containing acridine orange (AO, Sigma) and propidium iodide (PI, Sigma) was prepared in phosphate buffered saline (PBS). The final concentrations of the dual staining solution were 50  $\mu$ g/mL for PI and 10  $\mu$ g/mL for AO. A 100  $\mu$ L volume of cell suspension was mixed 1:1 with the dual staining solution. The resulting mixture was loaded onto a glass slide and cell number was determined with fluorescence microscopy (excitation by UV light), at 200x magnification.

Cells were scored into groups by virtue of their color and chromatin morphology. Cells exhibited green color corresponding to AO uptake, or red color corresponding to PI staining (i.e. cells which have lost membrane integrity). Green cells with dispersed chromatin were designated as viable; those with condensed chromatin were assigned as early apoptotic. Within the red cell group those with condensed chromatin fell into the “late apoptotic” category, while those with dispersed chromatin were determined to be necrotic.<sup>5</sup>

Three separate counts were performed for each analysis slide. Top, middle, and bottom regions of slide each, containing 100 cells each, gave a total of 300 counted cells for each sampling point. Viability results for each group were presented as a percentage of the total number of cells counted. For purposes of this study late and early apoptotic fractions were combined to a single “apoptotic” fraction.

**Guava Nexin Assay:** Samples of  $1 \times 10^5$  cells from induced and untreated cultures were harvested and centrifuged at 350 x g for 10 minutes at 4°C to remove culture medium and induction agents. Each cell pellet was resuspended in 40 mL of cold Nexin buffer (1X). Annexin V-PE (5  $\mu$ L) and Nexin 7-AAD (5  $\mu$ L) were added to each cell suspension and incubated on ice for 20 minutes to allow cells to stain. The sample volume was adjusted to a volume of 500  $\mu$ L by adding Nexin buffer (1X) before acquisition and analysis using the Guava PCA system. Apoptotic fraction was calculated as the percent of Annexin V-PE (+) cells.

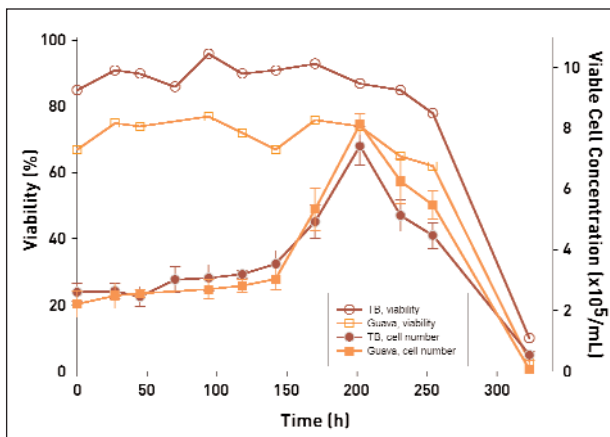
## Results and Discussion

### Cell Viability

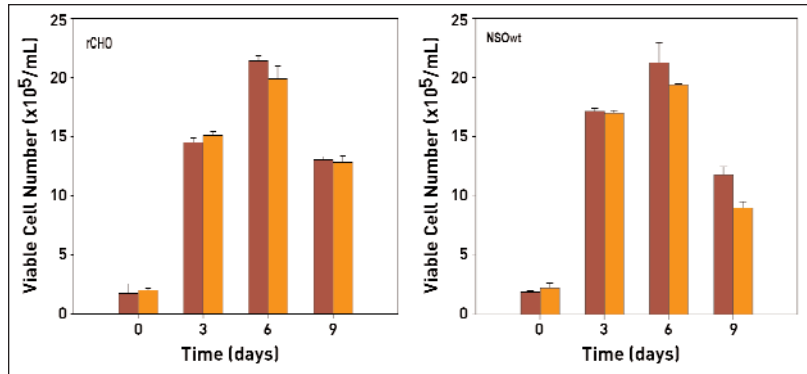
Measurements of viable cells and cell number during suspension cell culture were compared for trypan blue exclusion and Guava ViaCount methods. Figure 1 shows excellent correlation for viable cell number between the two methods, with no systematic over- or under-counting for rCHO cells. Investigators noted a great similarity in cell counts at each time point from both assays.

The objective of the batch experiment represented by Figure 1 was to compare survival of recombinant cells with that of parental lines. Growth rate was not of interest at this stage, hence the infrequent sampling. Loss of viability is surprising because cells were cultured for a time period consistent with normal culture conditions.

However, beginning at day 6 with NSOwt cells, ViaCount reported consistently lower viable cell count numbers than trypan blue. Figure 2 shows consistent correlation between the two methods for CHO320 cell number. However, ViaCount again consistently reported between 10% and 25% fewer viable cells than trypan blue. The rapid drop in viability at 220 hr for this batch experiment, shown in Figure 2,



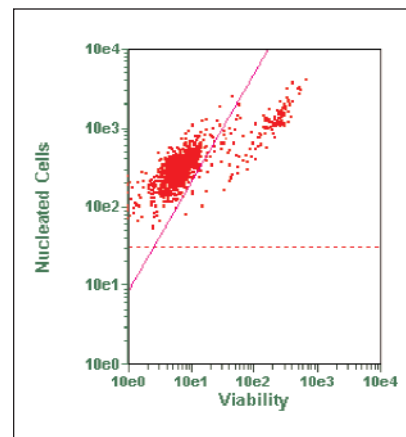
**FIGURE 2:** Comparison of Guava ViaCount and trypan blue exclusion for cell counts and viability. Measurements were taken on CHO320 cells cultivated in batch suspension cultures. Guava ViaCount consistently showed a lower viability count than trypan blue despite showing good agreement in total viable cell numbers.



**FIGURE 1:** Comparison of viable cell numbers using trypan blue (crimson) and Guava ViaCount (orange). Static batch cultures were carried out in serum-supplemented medium for (a) recombinant CHO cell line (rCHO) and (b) NSOwt cells.

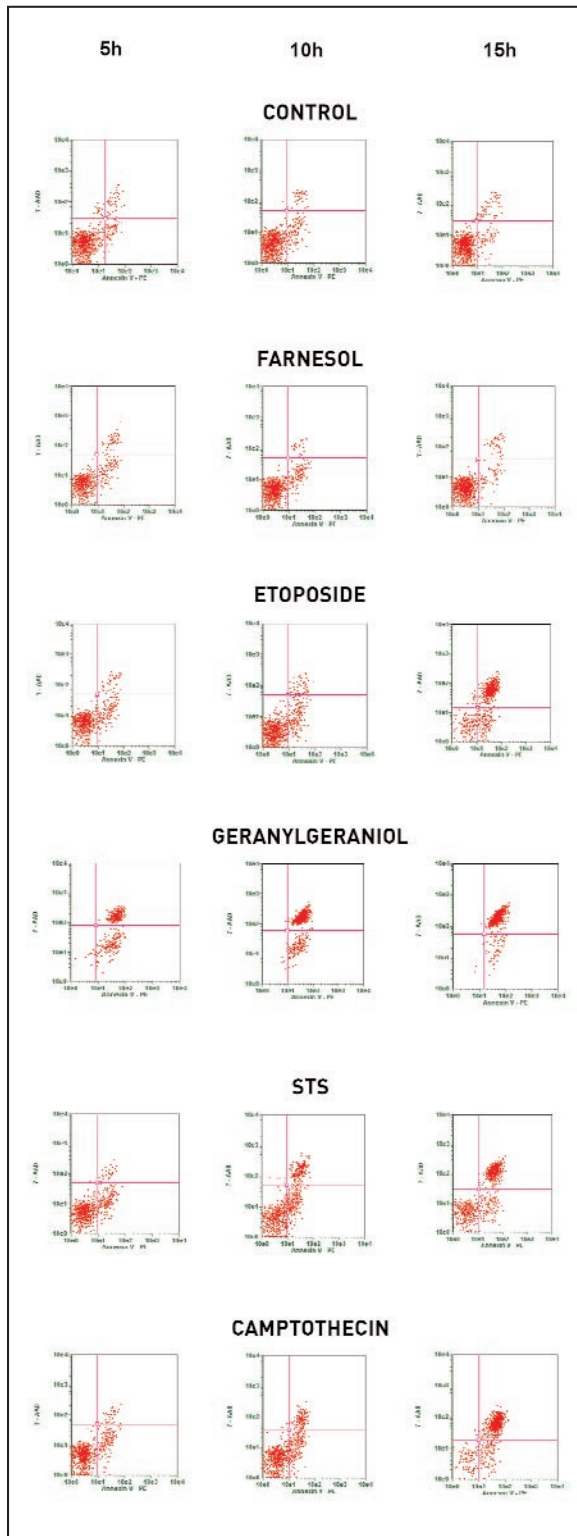
strongly suggests a decrease in vital nutrients in the medium and not due to external factors.

Lower viability values obtained with Guava ViaCount are related to this method’s ability to distinguish apoptotic cells from live cells (which trypan blue exclusion cannot do), and where the investigator sets the threshold for considering apoptotic cells “viable” or “nonviable.” In the Guava ViaCount assay, the staining pattern with the viability stain exhibits a range as shown in Figure 3. In the ViaCount assay, apoptotic cells exhibit diffuse, continuous staining behavior that resembles the expected behavior of viable cells at one end and dead cells at the other. In determining cell viability many researchers prefer to



**FIGURE 3:** Guava ViaCount Dot Plot of cells staining with intermediate levels of viability stain. Three distinct populations are evident on the plot: those highly staining with the viability stain (dead cells), cells that do not stain with the viability

dye (live cells-left of the purple gate), and the few cells intermediately staining with the viability dye (right of purple gate, left of highly staining cells). One of the ViaCount dyes stains only nucleated cells so all counts indicate true cell counts and exclude cellular debris.



**FIGURE 4:** Assessing an Apoptosis Induction Time Course with Guava Nexin. NSOwt. Cell cultures were treated with apoptosis-inducing drugs for the indicated time periods and assessed for apoptosis with the Guava Nexin assay.

gate live cells narrowly and include apoptotic cells with dead cells, which lowers viable cell counts relative to most staining assays. This effect is exaggerated when comparing ViaCount with trypan blue, which does not identify apoptotic cells at all. Since such cells do not stain, trypan blue cannot distinguish apoptotic cells, which will soon die, from healthy cells. Trypan blue therefore overestimates viable cell count. This effect is most apparent at culture viabilities below 50%.

Overestimation of viability with trypan blue has been noted previously, so the apparent under-reporting of viable cells is not an artifact of the ViaCount assay.<sup>6,7</sup>

### Guava Nexin Time Course Study

Figure 4 illustrates the Guava Nexin time course study on untreated control and treated NSOwt cells at various time points. Mid-exponential NSOwt cells were cultured in the presence of apoptosis inducers (staurosporine (STS), 4  $\mu$ M; camptothecin, 15  $\mu$ M; farnesol, 60  $\mu$ M; etoposide, 42.5 mM; and geranylgeraniol 50  $\mu$ M) over a 15-hour period. The lower-left quadrant in all graphs represents normal, viable cells. Data points in lower-right quadrant represent early-stage apoptosis, while cells in the upper-right quadrant are necrotic or in late-stage apoptosis. Control cultures were identical to treated cells except for non-addition of an apoptosis inducer. Figure 4 shows that the great majority of control cells are still viable at 15 hr., with a low level “background” apoptosis. Apoptosis levels were determined by Guava Nexin on a Guava PCA analyser.

Guava Nexin apoptosis detection is based on the calcium-dependent binding of Annexin V to PS translocated from the inner to the outer plasma membrane. Conjugation of phycoerythrin (PE) to Annexin V therefore allows detection of cells undergoing apoptosis by measurement of PE fluorescence. Similarly, dual staining with 7-AAD (a DNA counterstain) contained in the Guava Nexin kit identifies as necrotic those cells whose membranes have lost their integrity and become permeable.

Table 2, which relates to Guava Nexin results after 10 hr (Figure 4, Table 3), lists accepted modes of apoptosis induction for the inducers used in this study. For this time point analysis by fluorescence microscopy was also carried out from the same cul-

ture. Generally, an inducer that causes circa 50% apoptotic death within 10 hr is considered ideal. Hence the inducer of choice with this cell line is STS (Table 3). The relative lack of activity with farnesol could be explained by the fact the cells at this early stage of culture would be predominantly in G1 of the cell cycle, and therefore not susceptible to this agent. Conversely, the extent of apoptosis induced by geranylgeraniol suggests a mechanism radically different from those of the other inducers. However, we did not investigate these effects further.

**TABLE 2.** Apoptosis inducers and mode of action.

Inducer	Mode of Action
STS	Protein kinase inhibitor
Etoposide	Prevents association of DNA with DNA topoisomerase-I in DNA replication
Etoposide	Inhibits DNA re-ligation
Geranylgeraniol	Depletes intracellular anti-oxidants (reduced glutathione,GSH)
Farnesol	Acts in a similar fashion to geranylgeraniol but its activity is believed to be cell-cycle phase dependent (most active against S-phase cells)

## Conclusion

Guava ViaCount and Guava Nexin offer all the capabilities of conventional staining and cytometric or microscopic cell counting, but with additional benefits of near real-time analysis, lower cost, greater reproducibility, and shorter learning/training period. Compared with flow cytometry, the Guava PCA instrument is less expensive, smaller, and easier to learn. Because of the Guava PCA's higher level of automation and the reagents' specificity for intact apoptotic cells, the Guava system allows higher reproducibility, less opportunity for operator error, and significantly higher cell sampling (thousands of cells vs. hundreds).

In a bioprocess setting, Guava ViaCount provides a more realistic assessment of cell viability by measuring as apoptotic a significant number of cells that trypan blue counts as viable. Thus ViaCount offers process engineers a much earlier opportunity to alter cell culture conditions to maintain cell viability and productivity. Because Guava PCA and associated reagents operate in one-third to one-fifth the time of microscopy and flow cytometry, respectively, the Guava systems provide convenient, accurate, and rapid in-process cell viability measurements in systems under apoptosis-inducing stress.

**TABLE 3.** Comparison of apoptosis levels determined by Guava Nexin and fluorescence microscopy. Microscopy was done on cells dually stained with acridine orange/propidium iodide (AO/PI). Data was taken at 10 hours post-induction.

Culture Conditions	GUAVA NEXIN		FLUORESCENCE MICROSCOPY	
	Apoptotic Fraction (%)	Viable Fraction (%)	Apoptotic Fraction (%)	Viable Fraction (%)
Control	15.6	84.3	5.3	88.3
STS	43.7	55.8	25.8	69.4
Camptothecin	37.7	62.0	36.5	58.3
Farnesol	16.5	83.5	14.2	85.7
Etoposide	31.7	68.3	36.6	60.4
Geranylgeraniol	99.5	0.4	100.0	0.0

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## Product Notes

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