

# Comparative Study of the Time Dependency of Cell Death Assays

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## Abstract

Obtaining accurate viability counts is an integral part of any research conducted with mammalian cells. Different assays, however, test for different characteristics of cell death, occurring at different stages of the cell death process. Large differences in cell viabilities may be obtained from different assay types if cells are in an intermediate stage of programmed cell death. Therefore, a comparison of the time response of cell death, as measured by assays, is necessary.

In order to compare the relative time dependence of different assays, cell death was induced using streptozotocin (STZ) and anoxia (oxygen deprivation). Viability of the insulinoma cell line INS-1 cells was measured using three different types of assays: mitochondrial function assays, apoptosis indicating assays, and membrane integrity assays.

Results indicate that mitochondrial function assays detect cell death earlier than others, while membrane integrity assays detect cell death latest in the process. Results obtained from STZ-stressed cells are more reproducible than anoxia-stressed cells. Some assays yielded higher reproducibility; others resulted in less consistent results that may reflect the limitations of the assay.

## Introduction

As of 2002, 6.3% of the American population, or about 18.2 million people, were diagnosed with diabetes.<sup>1</sup> Recent medical studies have focused on transplanting insulin-secreting tissue isolated from human cadavers into diabetic patients as a new form of treatment. This process, however, puts an enormous amount of stress on the insulin-secreting cells. The success of this method is dependent upon the cells surviving the isolation and transplantation processes to restore normal blood glucose levels to the recipient. Therefore, an accurate, comprehensive assessment of tissue viability is essential prior to transplantation. The current method is propidium iodide (PI) staining, which is a membrane integrity test that identifies dead or damaged cells.

Viability assays differ in the way they mark cell death. Consequently, there is a distinctive time variation associated with individual assays. In this study, methodologies for various cell death assays were examined. One form of cell death is apoptosis, or “programmed cell death.” Apoptosis may be initiated by a stress, such as a poison, or it may be initiated within the cell. As cells undergo apoptosis, several characteristics emerge in stages: loss of mitochondrial function, caspase activation/DNA fragmentation, and loss of membrane integrity. Depending on which characteristics they assess, viability assays also fall into different categories: mitochondrial function assays, apoptosis-indicating assays, and membrane integrity assays. Since each of these assays tests for different stages in cell death, the time dependency and reported viabilities vary from assay to assay.

## Objectives

Our project aimed to elucidate the characteristic time-variation of eight assays. We tested their behavior when used with two different stresses. Three testable stages of cell death were examined: loss of mitochondrial function, physiological indications of apoptosis, and the loss of membrane integrity. The focus was to determine the extent to which assays that test for varying characteristics of cell death differ from one another. We hoped to use this information to compile a comparative plot of assay time response for eight assays. These plots will elucidate the stages of cell death for a given type of cell when induced with a particular stress.

## Assay Groups

### Mitochondrial Function Assays

Assays that test the mitochondrial function of dying cells include MTT, oxygen consumption rate (OCR), and adenosine triphosphate (ATP) assays. During the early stage of cell death, the H<sup>+</sup> ion gradient across the membrane relaxes, and the membrane potential of the mitochondria is lost as apoptosis proceeds.

The MTT is a tetrazolium compound, which is chemically reduced in cells to a colored formazan product. This conversion is presumably accomplished by NADPH and NADH produced by dehydrogenase enzymes in metabolically active cells.

The OCR assay determines the rate at which the cells in a given sample consume oxygen. Healthy cells consume oxygen at a specific rate that is unique for each cell line. By measuring the decrease in oxygen consumption rate with time, the number of viable cells can be calculated.

Finally, the ATP assay measures the concentration of ATP, which is proportional to the number of healthy, functional cells. Healthy cells produce an abundant amount of ATP, but as the oxygen consumption rate decreases during cell death, cells lose the ability to produce ATP, so the concentration of ATP decreases.

### Apoptosis-Indicating Assays

Apoptosis indicator assays recognize cells dying as a result of apoptosis rather than accidental forms of cell deaths such as heat shock.<sup>2</sup> Two types of apoptosis-indicating assays were tested: caspase activity and the externalization of phosphatidylserine (PS).

The caspase activity assay in the experiment used a fluorescent substrate to bind activated caspase. Caspases are enzymes that execute apoptosis in cells,<sup>3</sup> so the number of apoptotic cells can be quantified by measuring the fluorescent intensity of cells using flow cytometry.

Phosphatidylserine is a membrane component that is externalized early in apoptosis. Annexin V, another protein, naturally binds to phosphatidylserine.<sup>2</sup> Phosphatidylserine assays utilize Annexin V conjugated to a fluorophore to label apoptotic cells for flow cytometry.

## Membrane Integrity Assays

Membrane integrity assays detect the loss of membrane integrity during the later stage of cell death. Trypan Blue and Guava Viacount fall under this category; both assays consist of dyes that are impermeable to healthy cells, but can permeate through compromised membranes of dying cells. These dyes are either visible or fluorescent, and can be counted manually using a hemacytometer or with an automated flow cytometer, respectively.

FDA/PI is also a membrane integrity assay. Unlike Trypan Blue and Viacount, it is a double-assay. The cell sample is exposed to two fluorescent dyes: FDA, which stains cells with intact membranes, and PI, which characterizes cells with compromised membranes.

## Approach

Cell death was induced by streptozotocin and anoxia. Streptozotocin generates toxic levels of free radicals;<sup>3</sup> anoxia deprives the cells of oxygen. Since pancreatic tissue was not available for this study, INS-1 rat tumor cell lines were used instead. Stress-induced cells were then analyzed at various time points with the following assays:

### Guava Viacount

Sytox orange dye was diluted in PBS (phosphate buffered saline) and added to samples to dilute cell solution by 20X. Samples were then inserted in the Guava flow cytometer, which analyzed the viability of cells through flow cytometry and reported viability and total number of cells.

### Annexin

Cells were resuspended in 1X Nexin buffer solution, and 5  $\mu$ l of Annexin-V solution and 5  $\mu$ l of 7-AAD were added. To ensure the binding of the label, samples were incubated on ice. Finally, the 1X Nexin buffer was added and samples were analyzed in the Guava cytometer.

### Trypan Blue

Equal parts of cell solution and Trypan Blue dye were mixed. A hemacytometer was used to analyze the mixture under a microscope. The number of blue cells was counted, and fractional viability was calculated by dividing the number of clear cells by the total number of cells.

### MTT Cell Proliferation

Cell samples were added to the wells in a 96-well plate, and the MTT stock solution was added to each well. Plates were incubated for two hours at 37°C. To each well, SDS-HCl (10mL 0.01M HCL and 1g SDS) and DMSO were added. The plates were then incubated at 37°C for ten minutes, and placed in a plate reader to read absorbance at 540nm.

### Guava Multi-Caspase Detection Kit

After cells were resuspended in serum-free medium, they were diluted using 1X Apoptosis Wash Buffer. The cell suspension was combined in a microcentrifuge tube with 20X SR-VAD-FMK reagent, which was incubated

for an hour in a 37°C incubator. The cells were then washed with 1X Apoptosis buffer and centrifuged. The pellet was resuspended in 1X Apoptosis Wash Buffer, and Caspase 7-AAD was added. Samples were incubated for 10 minutes at room temperature, and analyzed using the Guava flow cytometer.

#### Oxygen consumption rate (OCR)

Cell samples were placed in a stirred tank chamber. An oxygen probe to the side of the chamber measured the dissolved oxygen concentration in the chamber. By calculating the rate of change in oxygen concentration, the oxygen consumption rate was determined.

A sample of viable cells will consume oxygen, whereas a sample of dead cells will not consume oxygen.

#### FDA/PI

Fluorescein diacetate (FDA) and propidium iodide (PI) were added to a cell sample, which was placed in a hemacytometer observed through a fluorescent filter. The cells that appeared bright green (FDA+) were counted and recorded as live cells. The cells were then observed through a rhodamine filter, and cells that appeared bright red (PI+) were counted to obtain the number of dead cells. To determine the total cell number, cells were observed under standard light.

## Results

### INS-1 cells with 5mM Streptozotocin

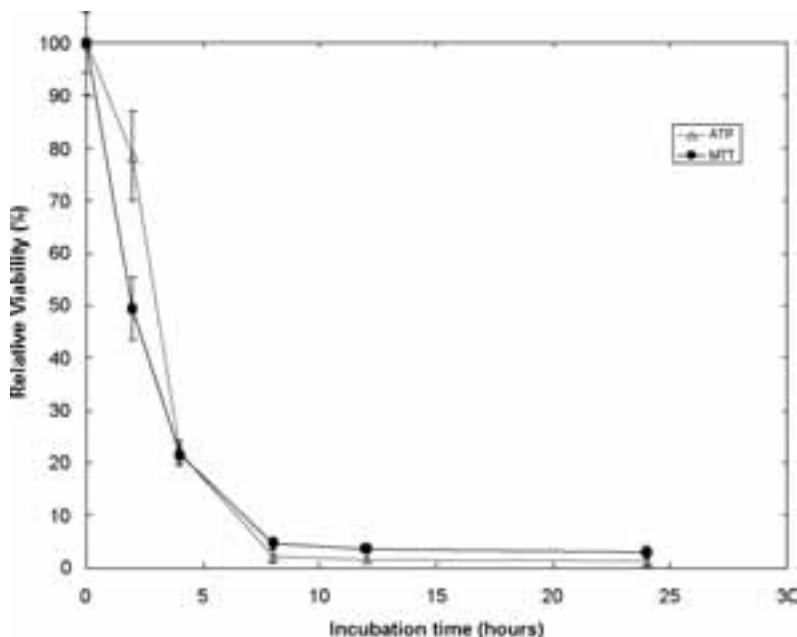
The results for streptozotocin can be broken down into five main categories: (1) mitochondrial function and cellular respiration assays, (2) apoptosis-indicating assays, (3) membrane integrity assays, (4) a comparison of all three assay types and (5) assay reproducibility. A description of the streptozotocin data follows in that order.

#### Mitochondrial Function and Cellular Respiration Assays

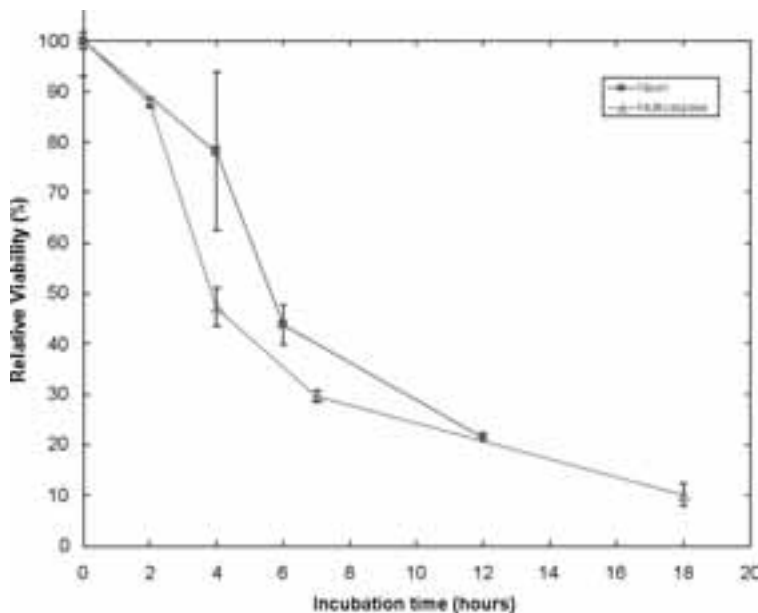
Figure 1 shows the results for the ATP and MTT assays run on May 4, 2004. The data show a strong correlation between the viabilities measured with both assays. While there is a significant difference between the two measured viabilities at the two-hour incubation (MTT=80%, ATP 50%), the rest of the viabilities are nearly identical.

#### Apoptosis Indicating Assays

The results for the two apoptosis-indicating assays, Guava Nexin Kit and Guava Multi-caspase, are plotted in Figure 2. All data points have been normalized based on the control. The Guava Nexin data points are from a set of samples run on April 6, 2004 and the Guava Multi-caspase data are from April 1, 2004. The graph indicates that Multi-caspase is an earlier detector of cell death compared to the Nexin. At an incubation time of 4 hours, the Nexin assay measures a viability of 80% while the multi-caspase assay predicts a much lower viability of 48%.



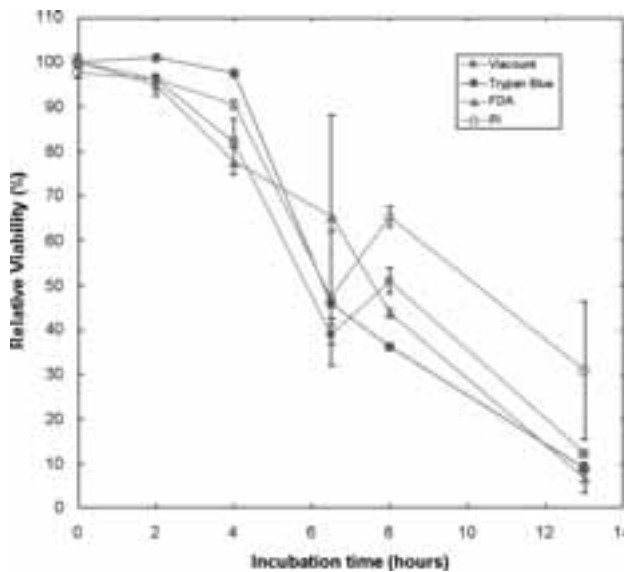
**Figure 1. ATP and MTT results for INS-1 cells stressed with 5mM streptozotocin on May 4, 2004.** The data show a strong correlation between the viabilities measured with ATP and MTT. While there is a significant difference between MTT and ATP at the 2-hour incubation (80% compared to 50%), all other time points overlap.



**Figure 2. Normalized Guava Nexin and Guava Multi-caspase results for INS-1 cells stressed with 5mM streptozotocin.** The Nexin and Multi-caspase assays were run on different days (Nexin data from April 6, 2004 and Multi-caspase data from April 1, 2004). Data are normalized so comparisons can be made. Multi-caspase appears to be an earlier indicator of cell death with viabilities consistently significantly lower than Nexin.

#### Membrane Integrity Assays

Four membrane integrity tests were used to measure the viability of the INS-1 cells stressed with 5mM streptozotocin. Figure 3 displays the data normalized by assuming 100% viability for control. The results show that all the assays give similar results for early incubation times. However, at the later incubation times of 8 hours and 13 hours, PI measures significantly higher values than the other three assays.



**Figure 3. Normalized Trypan Blue, FDA and PI results for INS-1 cells stressed with 5mM streptozotocin and run on April 22, 2004.** Data points are normalized by the control. All the assays result in similar values for the viability of the cell samples at early incubation times, while PI greatly overestimates the viability compared to the rest of the assays at later times.

One interesting aspect of the Guava Viacount is that the separation of live and dead cells is not well-defined. A reproduction of the Guava dot plots is shown in Figure 4. As shown in the seven-hour incubation sample, a distinct third population formed between what were considered the alive and dead populations. To distinguish between the living and dead populations, we used the control sample to set the boundaries as to what was dead and what was alive and used these settings on all the subsequent samples.

This third population also occurs with the FDA PI assay. Though both assays used fluorescent dyes, some cells fluoresced brightly while others displayed a weaker fluorescence pattern. Only the brightly colored cells were counted as FDA+ or PI+ in this case.

#### Combination

A comparison of all the assay types run on April 1, 2004 is shown in Figure 5. Relative viability is plotted (control was assumed to be 100% viable, and all other time points normalized against it). At about 12-13 hours, all assays indicate that the cells are dead. At early incubation times, OCR measures significantly lower viabilities than Viacount and Trypan Blue, and the Multi-caspase viabilities fall somewhere in between the two of them.

#### STZ Reproducibility

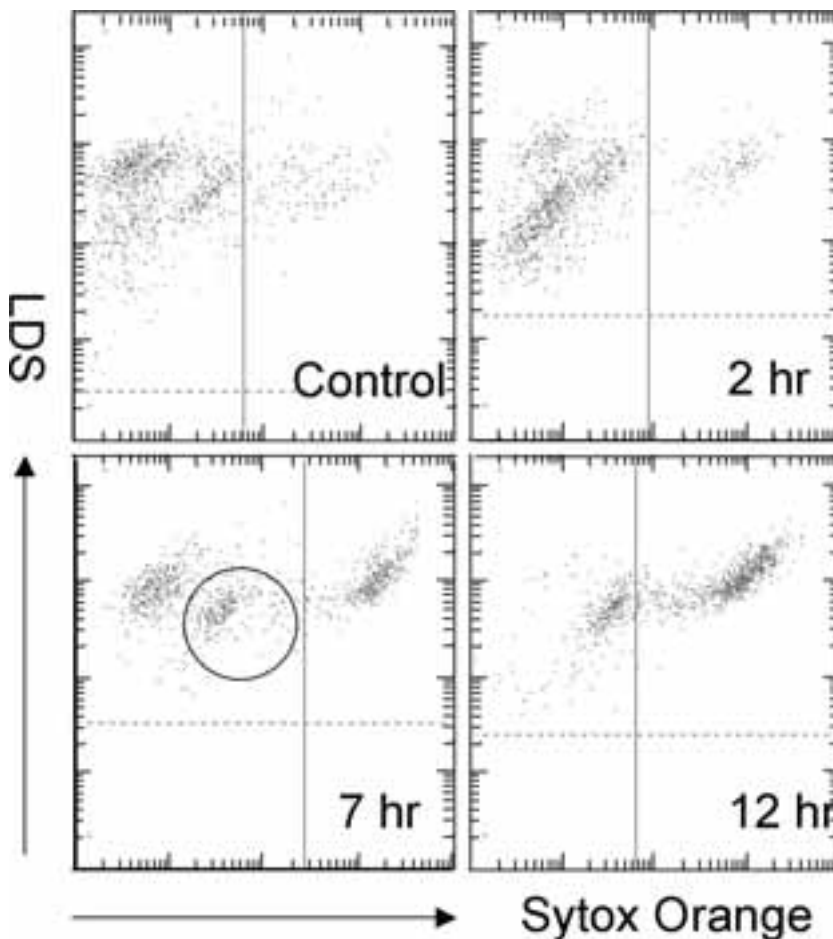
An example of the reproducibility of the experiments is shown for the Guava Viacount assay in Figure 6. The graph plots the results from five different experiments and shows the relative viabilities. For the most part, the data points coincide, suggesting reasonable reproducibility.

#### INS-1 cells stressed with Anoxia

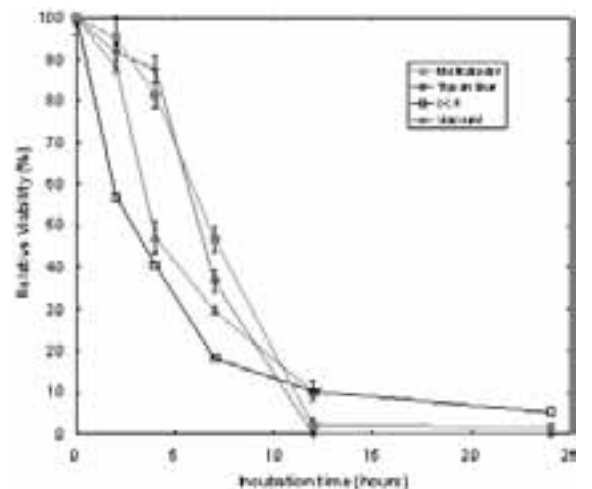
##### Mitochondrial Function and Cellular

##### Respiration Assays

As seen in Figure 7, OCR measures a higher viability than ATP, especially in later incubation times. Both curves, however, decrease sharply between the 12-17 hour incubation times, increase slightly at the 24-hour



**Figure 4. Guava Viacount flow cytometry plots.** The cells to the left of the vertical separator line are viable, while the cells to the right are dead. A distinct third population of cells is circled in the seven-hour incubation sample.



**Figure 5. Trypan Blue, Viacount, Multi-caspase and OCR results for INS-1 cells stressed with 5mM streptozotocin and run on April 1, 2004.** All assays indicate that cells are dead within 12 or 13 hours.



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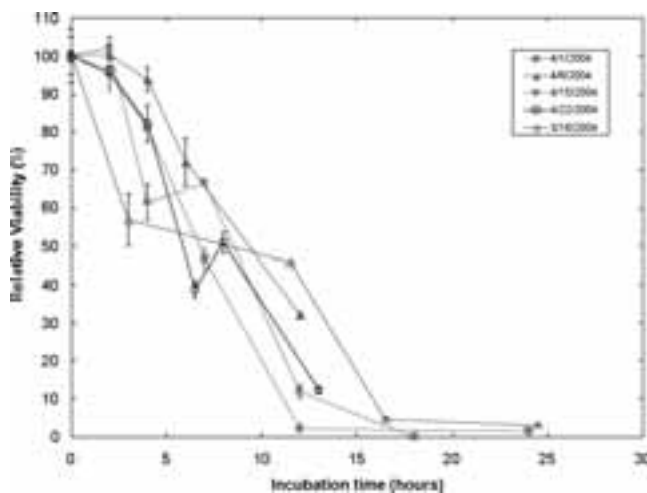
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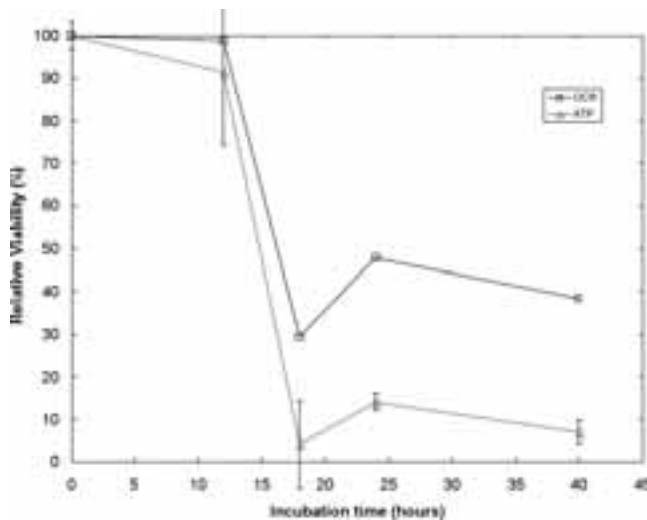
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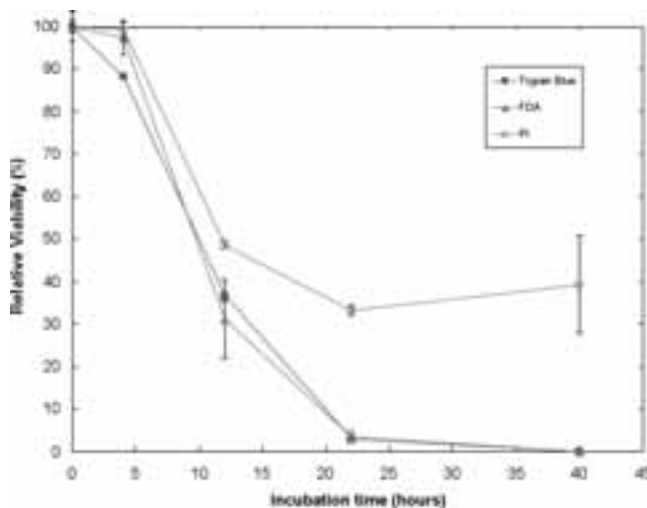
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**Figure 6. Guava Viacount reproducibility for INS-1 cells stressed with 5mM streptozotocin.** Reproducibility data are normalized by the value of the control ( $t = 0$ ).



**Figure 7. OCR and ATP results for INS-1 cells stressed with anoxia on May 6, 2004.** The results show that there is a significant difference in the viabilities measured with the two assays.



**Figure 8. Trypan Blue and FDA PI results for INS-1 cells stressed with anoxia and run on April 30, 2004.** Plot of relative viability indicates that PI results differ from the other assays.

incubation time point, then decrease again at the 40-hour time point.

#### Membrane Integrity Assays

Results obtained from trypan blue assay and FDA PI assay are plotted in Figure 8. While trypan blue and FDA follow each other closely, PI results diverge from the other two assays, especially at incubation times of 14 hours and longer.

#### Combination

Mitochondrial function assays and membrane integrity assays are plotted together in Figure 9. ATP predicts low viability at low incubation times, and the ATP curve falls sharply, while the other assays display a lag time of about four hours. PI yields a higher viability than the other assays, and most notably at higher incubation times.

#### Anoxia Reproducibility

In order to examine the reproducibility of data obtained from anoxia-stressed cells, results from different lab days are compared to each other. Figure 10 shows the comparison for trypan blue. The values for viability obtained on May 5 are significantly different from results obtained on April 30, revealing low reproducibility.

## Discussion

### INS-1 cells with 5mM Streptozotocin

Mitochondrial Function assays were performed on STZ-stressed INS-1 cells; results for the ATP and OCR assays are shown in Figure 1, and the viability measurements from the two assays follow each other closely. No significant lag time is observed, as both curves immediately display a decreasing trend at early times, and viabilities are less than 10% at the eight-hour incubation time point.

The apoptosis indicating assays, Guava Nexin and Guava Multi-caspase, are also compared to each other in Figure 2. Nexin consistently predicts a higher viability than the multi-caspase assay does, and the two assays diverge most significantly in the mid-time points between about two to twelve hours. It is important to note, however, that the data in Figure 2 are from two different lab days; the time-intensive nature of both assays made it impossible to run both assays on the same day. This means that the two assays were run on two separate cell batches, so the difference in viability measurement may reflect the difference in the cell samples, not the ability of the assays themselves; one cell sample may have contained more viable cells than the other.

The membrane integrity assays, on the other hand, were all run on the same day, and their results are compared in Figure 3. The relative viability curves for Viacount, FDA, and trypan blue follow each other closely, and most display an initial lag time of about four hours, which is most apparent in PI and trypan blue. The Viacount and PI assays result in an unexpected temporary increase at the eight-hour time point, but given the high standard deviation of the four-hour sample, the

eight-hour time point may actually be lower than the 4-hour time point. Overall, the viabilities display a decreasing trend. Compared to other membrane integrity assays, PI diverges the most in its viability measurement. Since PI is the most frequently used assay in islets, the inconsistency of PI with other membrane integrity assays is an interesting observation.

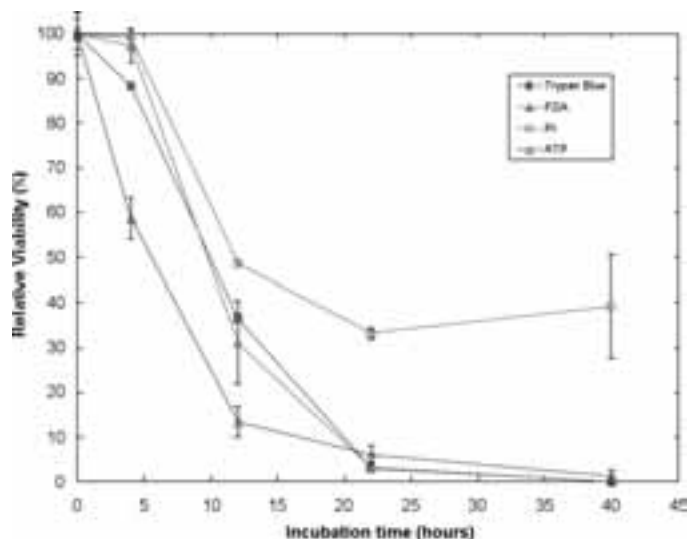
All three assay types are displayed on the same plot in Figure 4, in which the differences among different assay types emerge clearly. OCR is the earliest indicator of cell death: at the 4-hour time point, it reports a viability of 40%. Trypan blue and Viacount, which are both late indicators of cell death, report viabilities that are higher than 80% at that same time point. Their viability curves decrease sharply between the four- and twelve-hour time points, though, and result in lower viabilities at later time points than OCR. The viability curve for the multi-caspase assay, which is an apoptosis-indicating assay, falls in between the OCR and trypan blue curves, so the data in Figure 4 follow what was expected in the temporal response of these assays. The mitochondrial function assay is the earliest, the apoptosis indicating assays fall in the middle range, and the membrane integrity assays are last in measuring cell death.

Since many cell samples were assessed on different days, it was important to determine how reproducible data were from one day to another. Figure 5 displays the reproducibility of Guava Viacount assay. Viacount was chosen as an example because it was the most frequently used assay, and the reproducibility of the different assays was quite similar. The data are from five separate lab days, and as seen in Figure 5, the results obtained for INS-1 cells stressed with STZ appear to be quite reproducible, especially for a biological system. It is important to note that the graph that diverges the most from others was obtained at the earliest date (March 16, 2004), which may reflect that the team members' increased familiarity with the assay protocol throughout the term improved the data reproducibility.

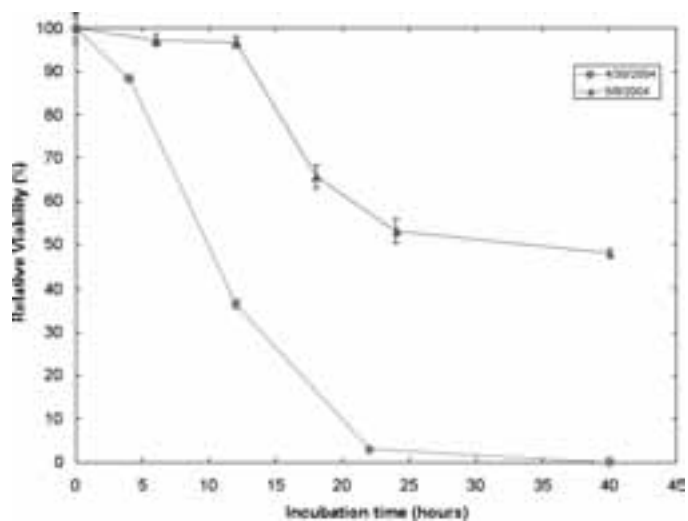
#### INS-1 cells with Anoxia

As seen with streptozotocin, the nine different assays used in this study tested for different characteristics of cell death that occur at different times during the process; therefore, the variability in the measured viabilities was dependant on the assay being used for the analysis. Of the assays run on cells stressed in anoxic conditions, most notable were the poor results of the OCR and PI assay.

As seen in Figure 7, OCR is not a practical assay for a stress such as anoxia. When compared to ATP as a cell viability measurement, OCR follows the same general trend, but the cell viabilities are overestimated at incubation times greater than 13 hours by almost 30%. Anoxia is the incubation of the cells in a low oxygen concentration environment. Testing the cells based on their oxygen consumption rate may not be realistic. Cells are able to survive in an anaerobic environment by



**Figure 9. Trypan Blue, FDA PA, and ATP results for INS-1 cells stressed with anoxia and run on April 30, 2004.** ATP reports a low viability value at early times, while PI consistently yields higher viability.




**Figure 10. Trypan Blue reproducibility for INS-1 cells stressed with anoxia.** At all incubation times, data obtained on different days report very different viabilities, which indicates poor reproducibility.

entering the anaerobic phase of cellular respiration. OCR measures the oxygen consumption of the cells, but there may be a time lag in between when the cells are reoxygenated and are able to switch from anaerobic respiration back to aerobic respiration.

PI is a membrane integrity assay that stains nucleic acid, both DNA and RNA. Results from PI diverged significantly from the other membrane integrity assays, Trypan Blue and FDA, especially at long incubation times. Neither Trypan Blue nor FDA stains DNA, which may be a possible explanation for this divergence. It is interesting that PI is one of the more commonly used tests for cell viability, but for both streptozotocin and anoxia, it consistently overestimated the values of cell viability and had the least amount of reproducibility.

A comparison of the two assay types, Figure 9, shows that there is a four-hour time lag between when ATP and



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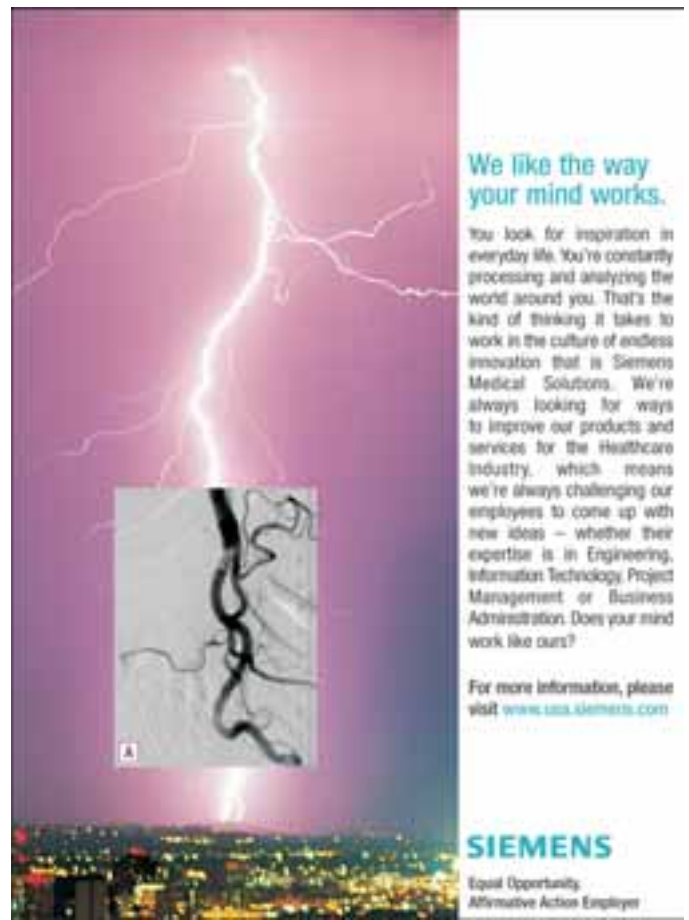
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the cell membrane integrity assays begin to detect cell death. As the loss of mitochondrial function is the first step in cell death, we expected results such as this.

Reproducibility for the trials with anoxia was generally poor as shown by Figure 10. The graph displays the result of the Trypan Blue assay run on two different lab days, April 30 and May 6. All the other assays showed a similar trend: at 40 hours, the cells from May 6 were still viable. With the Trypan Blue assay, one viability measurement was 50% and the other was 0%.

As mentioned before, cells can enter anaerobic respiration when deprived of the oxygen necessary for oxidative phosphorylation. The switch from the aerobic production of ATP to the anaerobic production is dependent on the amount of lactate dehydrogenase (LDH) present in the cells. LDH is the enzyme that is critical in the pathway of glucose to ATP in anaerobic respiration. It is believed that the amount of LDH present in a cell population is dependent upon passage time, or the number times a set of cells has been split after doubling in a cell culture flask. An improvement to this experiment would be to test for LDH along with cell viability.

The anoxia results showed that the type of assay used to analyze a cell sample plays an important role in the measured viabilities of the sample. Also of great importance is the effect the stress itself can have on the accuracy of the assay. With streptozotocin, ATP and OCR were the first indicators of cell death, consistently measuring viabilities less than the other assays. In the case of anoxia, the conditions of the stress interfere with the ability of OCR to work properly. Of all the assays tested, ATP yielded the best results.

## Conclusions and Recommendations

The assays generally performed as expected: mitochondrial function assays marked cell death first, and membrane integrity assays marked cell death last, while the results from apoptosis-indicating assays fell between results obtained from the other two. We also found that the conditions affected the performance of the assays. For example, OCR was a poor indicator of cell death for the anoxia trials because the stress deprived the cells of oxygen.

With regard to the reliability of assays, we found that ATP yielded reproducible results for both stresses and that OCR was a good indicator of cell death for cells stressed with streptozotocin. We recommend their use in further experiments. PI, which is commonly used to assess cell viability in islets, yielded consistently higher results for viability, making it a poor indicator of cell death. As shown by Figure 3, even among membrane integrity assays, PI is the latent indicator of cell death.

## Recommendations for Future Work

We recommend the use of ATP and OCR as the primary indicators of cell death. A complete reevaluation of the use of PI as a standard assay measure would also be illuminating. Finally, we recommend further study of the LDH and nucleosomal assays, which measures anaerobic respiration, and a signal of DNA fragmentation, respectively.

## Acknowledgments

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