Selection of an Optimal Cytotoxicity Assay for Undergraduate Research

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Abstract

Undergraduate research is a valuable tool to demonstrate both the dedication and time required to be a successful biologist. One area of research that has intrigued students over the last several years is cytotoxicity. However, at smaller undergraduate institutions, the time, training, and funding available for these research studies may be limited. Direct counting of cells is tedious and leads to mistakes, and although there are now several colorimetric toxicity assays, some have several steps and require near-perfect pipetting skills. To identify the most reproducible and affordable method(s) for undergraduate students to perform cell-based toxicity studies, we compared three colorimetric assays to counting viable cells directly. Using a breast cancer model system, students applied cantharidin to two different breast cancer cell lines, MCF-7 and MDA-MB-231, and performed MTT, resazurin, and crystal violet colorimetric assays or counted viable cells directly. We hypothesized that the MTT assay would be the most reproducible assay. Our results indicate that the crystal violet assay was not as reproducible as direct counting of cells, and therefore, not the best assay to use for toxicity tests. In contrast, the MTT and resazurin assays were highly reproducible and relatively low cost, and thus ideal assays for student research.

Key words: biology education; comparative study; higher education; cell viability

Introduction

Breast cancer is the second most common cancer in the United States, with about 230,000 new cases being discovered annually (www.cancer.org). Several breast cancer cell lines exist for research studies, including the well-characterized MCF-7 and MDA-MB-231 cells (Berthois, Katzenellenbogen, & Katzenellenbogen, 1986; Gupta & Kuperwasser, 2006; Harrell et al., 2006; St-Hilaire, Mandal, Commendador, Mannel, & Derryberry, 2011). We have used these cells in the past for testing of the toxicity of chemotherapeutics as well as pesticides (Kern & Schroeder, 2014; Jesionowski, Gabriel, Rich, & Schroeder, 2015; Florian, Mansfield, & Schroeder, 2016; Waszczuk & Schroeder, 2017) Additionally, other research has been published utilizing these as model systems for toxicity testing (Reardon et al., 1999; Ukpebor, Llabjani, Martin, & Halsall, 2011; Voborilova et al., 2011; Gurunathan, Han, Eppakayala, Jeyaraj, & Kim, 2013; Han et al., 2013; Gong, Goy, Olivo, & Yong, 2014).

Although a model system may be simple to select, the determination of the proper assays to monitor responsiveness can be difficult. There are several published and advertised cytotoxicity assays, examining both basic viability as well as metabolic activity. Henriksson et al. (2006) compared the amount of cell death observed using several assays, including cell counting, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tretrazolium bromide (MTT), crystal violet, and AlamarBlue (Henriksson, Kjellen, Wahlberg, Wennerberg, & Kjellstrom, 2006). MTT assays quantify the conversion of a yellow tetrazolium salt into purple formazan crystals using mitochondrial enzyme succinate dehydrogenase, which will only occur in viable cells (Riss et al., 2004; Niles, Moravec, & Riss, 2009; Sylvester, 2011). The AlamarBlue assay uses resazurin, a dye that shows both a colorimetric and a fluorometric change depending on cell metabolism, converting a blue dye to a pink color in the presence of active cells (Henriksson et al., 2006). Crystal violet is often used in microbial studies as a Gram stain. Due to the complexity of the staining process, it should detect only living cells. Dead cells are rinsed away through several washing steps, and the dye only stains the living cells after they have been fixed to a microplate. While this assay removes the chance of misconstruing increased or decreased metabolic activity as a direct consequence of increased or decreased cell number, it has several additional steps that can result in user error, especially for a student inexperienced in pipetting. These errors include washing away adherent live cells or not thoroughly washing away excess dye. By comparing the results of these differing assays, Henriksson et al. found that the observed cell viability in cell line LU-HNxSCC-7, which originated from a head and neck squamous epithelia carcinoma, was dependent on

which assay was used (Henriksson et al., 2006).

To compare these assays, we wanted to utilize both healthy, untreated cells, as well as cells exposed to a toxin; thus, we would be comparing the assays in a method similar to how undergraduates would be using them for data collection in a toxicity-style assay. The chemotoxin used in this study was cantharidin, which is produced by the blister beetle and is known for its anti-tumor affinity (Efferth et al., 2005). Cantharidin induces apoptosis through the p53 mechanism either intrinsically by causing mitochondrial release of cytochrome C, or extrinsically via activation of the caspase cascade (Chang et al., 2008). Cantharidin also causes oxidative stress that provokes DNA damage (Li et al., 2010). We have previously shown that cantharidin is a more potent activator of cell death than other common chemotherapeutics using an MTT assay (Kern & Schroeder, 2014).

Historically, crystal violet and MTT assays have been well-published, with fewer studies using AlamarBlue. Based on past experiences in our research group, we hypothesized that there would be issues with reproducibility in the more complex assays (crystal violet), but those requiring minimal pipetting would show fewer differences between replicates. Additionally, our goal was to determine which colorimetric assay best represented the number of viable cells determined by direct counting.

Materials and Methods

Cell Culture and Treatment

MCF-7 cells were maintained in MEM media with 5% calf serum, whereas the MDA-MB 231 cells were maintained in DMEM media with 10% newborn calf serum. During plating, cells were removed from a T-75 flask using trypsinization. Cells were washed twice with HBSS to remove residual serum proteins, then treated with 1 ml 0.05% trypsin for 5 minutes at 37ºC. Cells were removed from the flask using physical perturbation. Media (10 ml) was added to the suspended cells, and cells were evenly transferred into a 96-well plate with 100 µl suspended cells per well or to a 6-well plate with 1.5 ml of suspended cells per well. Cells were allowed to adhere to the microplate for approximately twenty-four hours before treatment with toxin began. For the cytotoxicity studies, cells were treated with either 500 nM to 50 µM cantharidin or 1 µM to 100 µM cantharidin. Untreated cells were replenished with fresh media on the day of treatment. After the treatment exposure for 48 hours, the viability of the cells was quantified by cell counting or by using colorimetric assays with MTT, resazurin, or crystal violet.

Viability Assays

As a control assay, we counted viable cells directly without colorimetric staining procedures. Cells were treated in a 6-well plate. Following treatment, cells were washed with twice with HBSS. Trypsin (0.05%, 0.5 ml) was added to each well and cells were incubated for 5 minutes at 37ºC. Detachment from the wells was determined visually and cells were pipetted into a 15-ml conical. HBSS (4.5 ml) was added to dilute residual trypsin and live cells were counted immediately on a hemocytometer. Eight squares of cells were counted for each treatment and averaged within each individual experiment.

In the crystal violet staining method, media was removed and 100 µL of 50% v/v ice-cold methanol was added to each well for 10 minutes to fix cells. After 10 minutes, the methanol was removed, and 50 µL of 1% w/v crystal violet was added to each cells for staining. After 10 minutes, the dye was removed and cells were rinsed twice with water to wash away the excess dye and any poorly-adhered cells. The dye was dissolved in 1% SDS, and the amount of stain absorbed by the live cells was quantified with a microplate reader at a wavelength of 540nm. Viable cells were quantified by normalizing the absorbance readings to the untreated control cells, set at 100% viability.

For the MTT assay, 10 µL of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) was added to the wells and placed in the incubator for 3 hours. After 3 hours of growth, the media was removed and 200 µL of DMSO was added to each well to dissolved the crystals (Riss et al., 2004). The plate was then read on a microplate reader at 570nm. Viable cells were quantified by normalizing the absorbance readings to the untreated control cells, set at 100% viability.

For the resazurin assay, 20 µL of 0.15 mg/ml resazurin was added to each well. After three hours of incubation, the plate was read at 570 and 595 nm on a microplate reader. Viable cells were quantified by subtracting the absorbance reading at 595 nm from the reading at 570 nm, and normalizing to the untreated control cells, set at 100% viability.

To ensure consistency during direct comparisons, all three colorimetric assays were run by the same undergraduate student, together on a single microplate. All experiments were run in triplicate on each plate, and three plates on different days were used for each cancer cell line. Results were normalized to the control within each replicate. Differences in viability for compiled data were confirmed using a between-subject test and ANOVA with a Fisher's Least Significant Difference post-hoc test using SPS (IBM SPSS Statistics 21, IBM Corp., Aramonk, NY, USA). Significant variation from controls was .

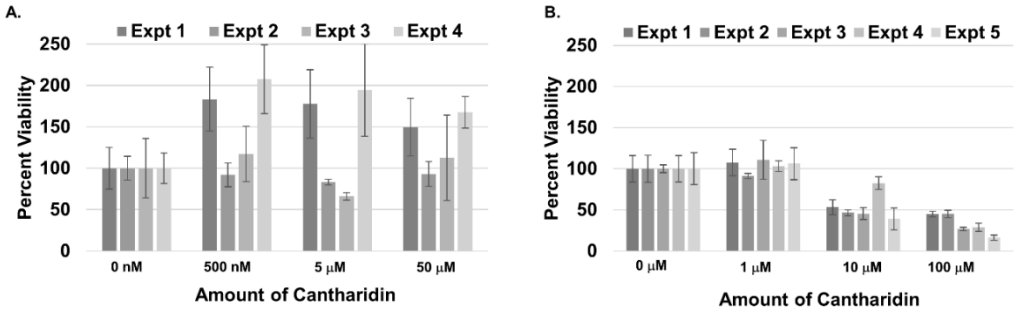

Fig. 1. Crystal Violet or MTT detection of viability of MDA-MB-231 breast cancer cells after toxin treatment. All results were normalized to the media control (0 nM/μM treatment) for each individual experiment (Expt). Error bars represent standard deviation amongst replicates in a single experiment. A. Cells were treated in quadruplicate with 500 nM to 50 μM cantharidin for 48 hours followed by staining with crystal violet. Stained cells were quantified by reading absorbance at 540 nm in a microplate reader. Results represent four separate experiments. B. Cells were treated in triplicate with 1 μM to 100 μM cantharidin. After 48 hours, cells were stained using MTT, and the formazan crystals were dissolved in DMSO prior to reading absorbance at 570 nM on a microplate reader. Results represent five separate experiments.

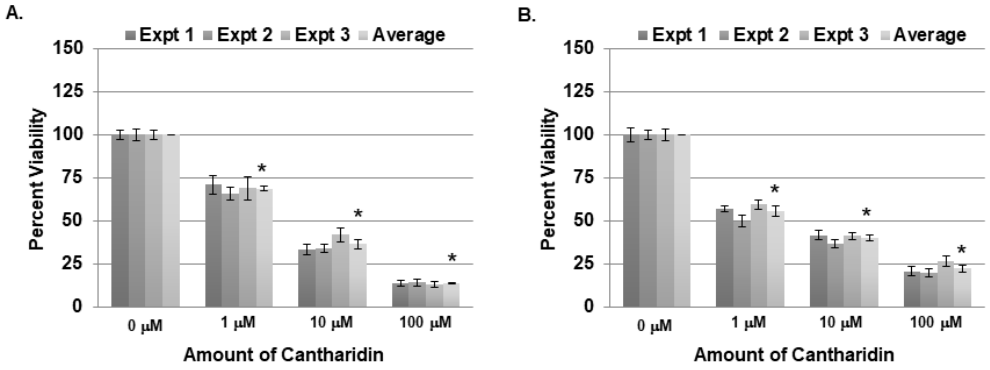

Fig. 2. Cell counting assay to determine viability of breast cancer cells after toxin treatment. MCF-7 (A) or MDA-MBA-231 (B) breast cancer cells were treated in triplicate with 1 μM to 100 μM cantharidin. After 48 hours, cells were removed from the wells using trypsin, and eight sets of viable cells per well were counted manually using a hemocytometer. All results were normalized to the media control (0 nM/μM treatment) for each individual experiment (Expt). Error bars represent standard deviation amongst replicates in a single experiment. Results represent three individual experiments run in triplicate; error bars indicate SEM. Statistically different viability compared to the control is indicated (*, $p<0.05$).

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Results

One of the main focuses of our research lab is studying the toxicity of natural and man-made chemotherapeutics using a human breast cancer cell model. However, one of the challenges with undergraduate research is being able to discern when discrepancies from hypothesized results are due to true scientific data versus user error. Consistency with pipetting can be a difficult skill for undergraduate researchers to master; we have previously had confusion about the validity of data sets when repetitions of assays look completely different (Figure 1A). Early student researchers in our lab utilized crystal violet-based colorimetric assays (Rich et al, 2012), but results were sometimes contradictory from week to week. For example, during the time that one student showed large variations in viability (Fig. 1A), a second student was achieving high reproducibility using an MTT assay (Fig. 1B, individual data sets from

averaged data previously published in (Kern & Schroeder, 2014)). Subsequent research using MTT or resazurin assays were much more reproducible for all students involved (Waszcuk & Schroeder, 2017; Siegfried & Schroeder, 2018).

Due to these large variations between replicates in many crystal violet assays, we wanted to determine if there was an optimal assay that undergraduate students could utilize with both reproducibility and reliability. Thus, a single undergraduate student compared three colorimetric assays to a direct counting of viable cells. Since we had recently published on the high toxicity of cantharidin using an MTT-based assay (Kern & Schroeder, 2014), we utilized that same toxin in this comparison study and expanded our work to compare these assays in two distinct breast cancer cell lines.

As our assay control, the undergraduate researcher performed a cell counting assay. Two different breast cancer cell lines were plated into a 6-well microplate to facilitate easier removal than from a 96-well plate. After treatment with cantharidin for 48 hours, cells were removed by trypsinization and counted using a hemocytometer. Figure 2 shows the reduction in live cells for cantharidin-treated MCF-7

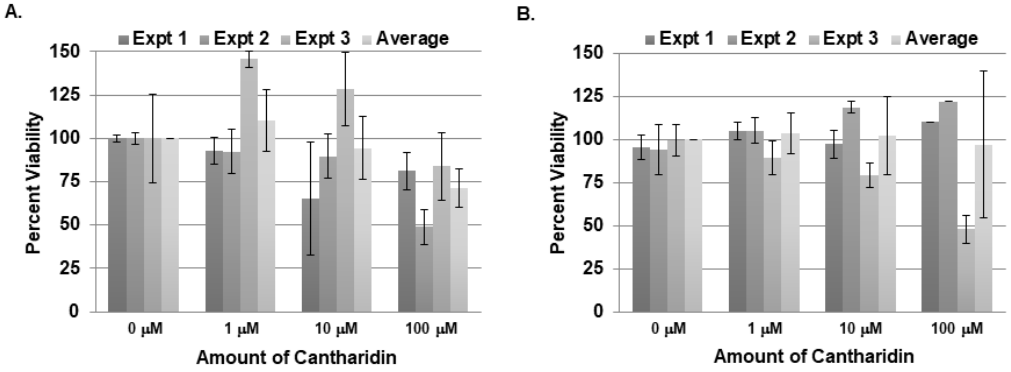

Fig. 3. Crystal violet colorimetric assay to determine viability of breast cancer cells after toxin treatment. MCF-7 (A) or MDA-MBA-231 (B) breast cancer cells were treated in triplicate with 1 µM to 100 µM cantharidin. After 48 hours, cells were fixed and stained with crystal violet. Stained cells were quantified by reading absorbance at 540 nm in a microplate reader. All results were normalized to the media control (0 nM/µM treatment) for each individual experiment (Expt). Results represent three individual experiments run in triplicate; error bars indicate SEM.

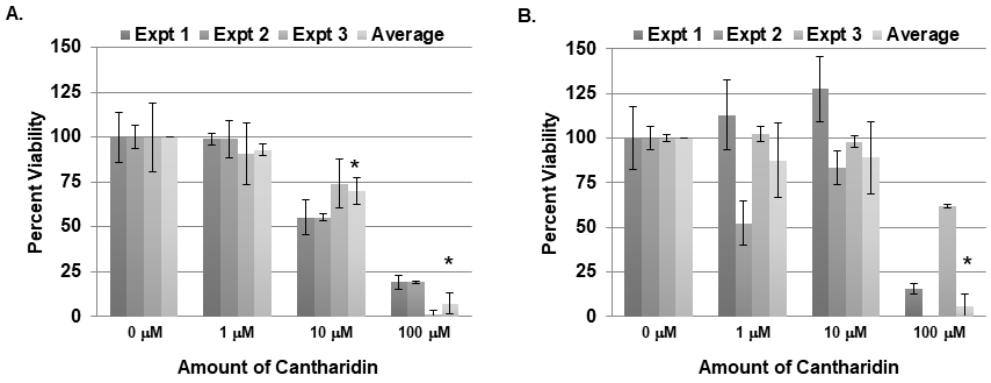

Fig. 4. MTT assay to determine viability of breast cancer cells after toxin treatment. MCF-7 (A) or MDA-MBA-231 (B) breast cancer cells were treated in triplicate with 1 µM to 100 µM cantharidin. After 48 hours, cells were stained using MTT, and the formazan crystals were dissolved in DMSO prior to reading absorbance at 570 nM on a microplate reader. All results were normalized to the media control (0 nM/µM treatment) for each individual experiment (Expt). Results represent three individual experiments run in triplicate; error bars indicate SEM. Statistically different viability compared to the control is indicated (*, $p<0.05$).

We then tested the colorimetric assays in a single microplate. As we had previously observed during initial (and less reproducible) studies, the crystal violet assay did not indicate cell death (Fig. 3A). As the concentration of cantharidin increased, no consistent reduction in viability was observed in either breast cancer cell line tested. Results from individual experiments showed large variations with over a 60% range in viability in some treatments in MCF-7 cells, such as for the 10 µM cantharidin treatment (Fig. 3A). Only one of the 100 µM cantharidin treatments in MDA-MB-231 cells showed cell death (Fig 3B).

In contrast, both the MTT and resazurin assays (Figs. 4 and 5) showed decreases in viability after treatment with cantharidin. For MTT assays, low variations between experiments were observed in MCF-7 breast cancer cells (Fig. 4A). Significantly less viability was observed in the averaged data for both the 10 µM and 100 µM cantharidin treatments. Less consistent results were observed for MDA-MB-231, although all three individual experiments showed lower viability with the 100 µM cantharidin treatment, and the averaged data was statistically less than the control for the 100 µM cantharidin treatment (Fig. 4B). Similar results were observed with the resazurin assay (Fig. 5). However, both cell lines showed more reproducible results for all three individual experiments.

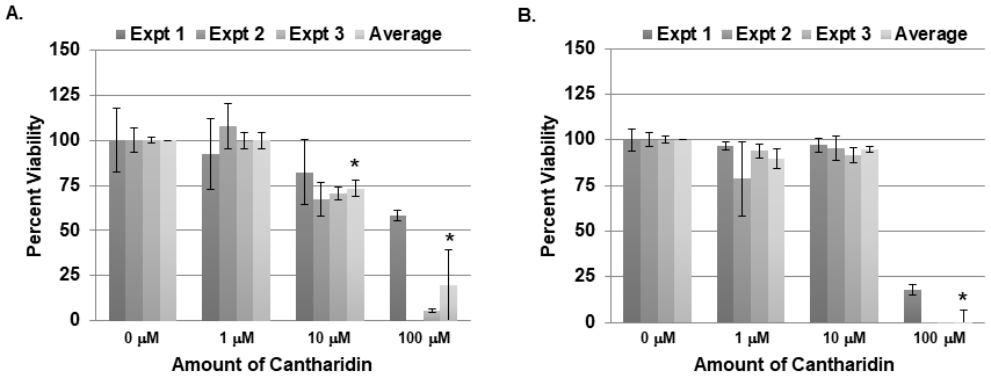

Fig. 5. Resazurin assay to determine viability of breast cancer cells after toxin treatment. MCF-7 (A) or MDA-MBA-231 (B) breast cancer cells were treated in triplicate with 1 µM to 100 µM cantharidin. After 48 hours, cells were stained using resazurin, and viable cells were quantified by reading absorbance at 570 nm and 595 nm. All results were normalized to the media control (0 nM/µM treatment) for each individual experiment (Expt). Results represent three individual experiments run in triplicate; error bars indicate SEM. Statistically different viability compared to the control is indicated (*, $p<0.05$).

Discussion

When determining whether compounds affect cell viability or cause cell death, several methods are available. One can test for the structural integrity of the nucleus or mitochondrial membrane, examine ATP and ADP levels, or stain cells using a variety of methods. However, in many research labs funding can be difficult to obtain, especially when performing research at smaller institutions, where performance earning is still as highly valued as at larger, Tier 1 research institutions. In these situations, it may not be feasible or affordable to purchase a cell counter, fluorescence microscope, or tritiated thymidine, much less train undergraduate students in their use. In these cases, other options must be examined.

Although a direct counting of cells may be the most accurate, there are drawbacks to this method. First, counting of the cells requires either counting of the entire well, or removal of cells via trypsinization. This can result in some damage to the cells, reducing the amount of counted cells. While this may be accounted for in normalization to a control (assuming the same amount of damage occurs in all wells), it does require more media and disposable plasticware, adding a cost increase to the experiment. It would take sixteen 6-well microplates to test the same number of wells as in a 96-well plate. For stock-brand tissue-culture treated disposable plates from many suppliers, this can increase the cost of a single experiment from less than $2.50 to greater than $20. Additionally, trypsinization may not yield adequate dissociation for all cell types and may, in fact, alter the cells in the subculture (Chaudhry, 2008; Park et al., 2008; Zhang et al., 2012). Beyond this, there is a 29-fold increase in surface area in a 6-well plate; this requires more cell culture resources per treatment. We have routinely assisted colleagues in assessing chemical constructs made in very low quantities that we would be unable

to perform replicates on were we to use only the cell counting method (unpublished data).

Thus, colorimetric assays have become a go-to method for testing cell proliferation and death. throughout the years, several experiments have used crystal violet as an effective method for testing of toxins and chemotherapeutics (Henriksson et al., 2006; Geserick et al., 2009; Feoktistova, Geserick et al., 2016a; Feoktistova, Wallberg et al., 2016b). Our data refutes those experiments. In our experience, the crystal violet assay may not be as effective for toxicity testing, since when using the crystal violet assay, much less cell death was quantified than with the MTT and resazurin assays in adjacent wells. Possible sources of error, with the crystal violet assay in particular, may include scraping or blowing the cells off the bottom of the plate with the pipette tip. Another possible source of error may include overpopulation of cells in the flask while growing. Also, the crystal violet assay tests how many viable cells are left after treatment, but staining is done with filtered dye after first fixing the cells. Rinsing is a key step to accurate readings. If proper rinsing of debris and dead cells was not conducted before dyeing the cells, or any precipitated dye remained in the wells after staining, an overestimation of cell viability would have resulted.

To eliminate some of the sources of variation (pipetting and rinsing errors), MTT and resazurin-based assays could be utilized. Mueller et.al. indicated that MTT may, in particular, serve as a preferred method for high-throughput screening of cytotoxic agents (Mueller, Kassack, & Wiese, 2004), while Borra et.al. showed that resazurin use can provide accurate assaying of mitochondrial activity at a low cost to the researcher (Borra, Lotufo, Gagioti, Barros Fde, & Andrade, 2009). In our own work, the cost of an MTT assay is approximately $4 per plate, with over half of that cost due to the disposable plasticware. Resazurin assays are even less expensive, as the resazurin salt is low cost (less than $30 per gram) and less than 3 mg is used per plate. However, there are several limitations to these protocols. While the resazurin assay does not require any rinsing of cells and thus may be able to be used for suspension cultures, both the crystal violet and MTT assays do require at least one rinse step. For a suspension culture, this would require repeated pelleting of the cells, which would risk damage or loss of cell material. Thus, these assays are better suited for adherent cells. As an additional complication, the type of toxicant being studied may also interfere with these reagents. Angius et. al. demonstrated that MTT has an affinity for lipids, and thus any toxicants applied through a liposome method may interfere with proper absorbance of MTT by the cells (Angius & Floris, 2015). MTT has also been shown to interact with fat-soluble compounds such as flavonoids and the vitamin E isomer α-tocopherol (Peng, Wang, & Ren, 2005; Lim, Loh, Tring, Bradshaw, & Allaudin, 2015). Free thiol groups can also reduce MTT to formazan (Shoemaker, Cohen, & Campbell, 2004).

Unlike the crystal violet assay, both the MTT and resazurin assays in both MCF-7 and MDA-MB 231 cell lines were able to indicate a reduction in metabolic activity, and this was attributed to a concurrent reduction in viability. This was highly reproducible, especially within MCF-7 cells. As an added benefit to undergraduate research, the resazurin assay method has fewer steps than the MTT assay method. For inexperienced pipettors, the resazurin assay may be ideal. However, there is one drawback to this protocol. Since the dye is added directly to the media, any components present in the media or in any toxins being tested could interfere with the colorimetric assay (O'Brien, Wilson, Orton, & Pognan, 2000; Simeonov & Davis, 2004). This includes coloration within the additives as well as pH variations that could alter the resazurin dye. In these cases, a more optimal assay may be the MTT assay, which still exhibited high reproducibility. We were faced with this issue within our own recent research, where the toxins being studied (essential oils) were colored and created a color artifact that interfered with the resazurin assay (Siegfried & Schroeder, 2018). Thus, an MTT-assay was utilized as all of the colored oils were removed from the wells prior to the addition of the MTT dye.

Although these methodologies are generally accepted as reflecting the number of live cells present, they do this through an assumption that metabolic activity remains constant across that cell population. Both resazurin and MTT assays rely on the conversion of the dye through increased redox activity. An increase in the amount of conversion may be accomplished not only through the presence of more cells, but also through a rise in the metabolic activity of a stable cell population. The majority of the redox activity involved in the conversion is attributed to mitochondrial NADH and NADPH, but these coenzymes are also able to reduce the dyes extracellularly as well as outside the mitochondria or even external to the cell itself (Bernas & Dobrucki, 2002; Uzarski, Divito, Wertheim, & Miller, 2017). Likewise, the presence of redox inhibitors can result in a drop in formazan production even if cellular levels remain constant (Stepanenko & Dmitrenko, 2015; Shenoy et al., 2017). Resazurin has been indicated previously as a more reproducible, and thus more accurate, determinant of cell viability (van Tonder, Joubert, & Cromarty, 2015). However, nonlinear growth of cells can result in inaccurate resazurin

correlations (Mallick, Scutt, Scutt, & Rolf, 2009; Quent, Loessner, Friis, Reichert, & Hutmacher, 2010; Rampersad, 2012). Thus, while these colorimetric assays can still be utilized as a general means of determining viability, conclusive changes in cell number may be determinant upon the assay conditions. We have observed these differences in our own study, where a direct counting of cells identified a reduction of viability under much lower cantharidin concentrations than was indicated by the metabolic assays (compare Figure 2 to Figures 4 and 5). Thus, we recommend that both resazurin and MTT are still feasible and economically preferential options for determination of cell viability in undergraduate research projects, as much of the literature and company advertisements claim. However, if financially practical, a concomitant counting of cell number would add to the study, and might allow students to tease out the differences between viable and metabolically active cells.

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