Sensitive cell viability assay for use in drug screens and for studying the mechanism of action of drugs in *Dictyostelium discoideum*

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In order to increase the effectiveness of Dictyostelium discoideum *as a lead genetic model for drug discovery, a luminescence-based assay has been adapted and standardized for sensitive and rapid cell viability measurements. The applicability of the assay was demonstrated by measuring the cytotoxicity of several drugs in wild-type and mutant cells. The robustness and ease of the assay demonstrate that it can be used in high-throughput applications such as drug or mutant screens. Conclusions from these studies are applicable to evaluating cell viability assays in other systems as well.*

INTRODUCTION

Drug resistance is a major obstacle in chemotherapy and the continuing subject of extensive research. Many cancer patients who are treated with chemotherapeutic drugs will exhibit resistance to the drugs (whether inherent or acquired during the course of treatment), and since many of the drugs are used at their maximum medically allowable dose, even 1.5- to 2-fold resistance presents a serious problem. Thus, a great deal of research is focused on understanding the mechanism of action of the drugs, with the goal of identifying new targets for therapy, as well as enhancing the efficacy of existing drugs. Often such studies can be conveniently performed in experimental model systems, which offer powerful genetics and accessible biochemistry.

Any approach to understanding drug resistance relies on determining cell survival after different treatments, and it requires a rapid assay that can accurately measure cell viability over a wide range of cell concentrations and, at the same time, is sensitive enough to detect even a small number of surviving cells. This has been a significant challenge in performing large-scale genetic screens in a variety of model systems.

Dictyostelium discoideum is being used increasingly as a primary system for drug discovery and for studying the mechanisms that underlie the response to drugs (1,2), including anticancer drugs (3,4). Traditionally, cell viability was measured by plating and counting the plaques resulting from viable cells. The method is labor-intensive and requires considerable experience on the part of the investigator to routinely obtain quality data. Even when using a modification that significantly reduces cost and time (5), this method is not practical for assaying large numbers of samples, as is the case in a high-throughput drug screen. Clearly, a faster robust biochemical assay would benefit such studies.

In the following study, we describe the adaptation of a commercial luciferase-based assay to determine viability in populations of *D. discoideum* cells. In this assay, survival is determined by assaying for the amount of ATP contained within living metabolically active cells. We describe the optimization and necessary conditions that allowed its use in *D. discoideum* and show that the assay offers the sensitivity, reproducibility, and ease that are required to perform large-scale screens in this organism. Moreover, it has allowed us to gain preliminary insights into the different mechanisms of cytotoxicity by different drugs.

MATERIALS AND METHODS

Drug Treatments and Viability Assays

96-Well plates. Aliquots (90 µL) of logarithmically growing *D. discoideum* cells in HL-5 axenic medium (7 g yeast extract, 14 g proteose peptone, 0.48 g KH_2PO_4, 0.5 g Na_2HPO_4, per liter, pH 6.5) (6) were placed in 96-well opaque white plates (Matrix Technologies, Hudson, NH, USA). Ten microliters of the drug being tested (in the indicated solvent) were added at the indicated concentrations and incubated at 22°C as described for each experiment. Ten microliters of the corresponding solvent were always added to the untreated controls. Following incubation with the drug, 100 µL CellTiter-Glo™ or BacTiter-Glo (Promega, Madison, WI, USA) were added, and the plates were covered and shaken for 30 min at room temperature. Luminescence was measured in a Veritas™ Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA). Survival was calculated as the percentage of relative luminescence units (RLU) of the treated versus untreated culture. Error bars represent standard error, which was calculated using statistical tools in Microsoft® Excel®. *P* values were calculated using Student's *t*-test. Number of replicates is stated in each experiment. All drugs were from Sigma-Aldrich (St. Louis, MO, USA). Cisplatin was prepared as a 1 mg/mL 3.3 mM solution in aqueous Pt buffer (3 mM NaCl/1 mM $NaPO_4$, pH 6.5). Dimethyl sphingosine (DMS) was prepared as a 1 mM solution in dimethyl sulfoxide (DMSO), and 4-nitroquinone (4NQO) was prepared as a 2 mg/mL solution in DMSO. Both DMS and 4NQO were diluted in aqueous solution prior to adding to the cultures. The final DMSO concentration in cell cultures never exceeded 2%, which is not toxic to *D. discoideum.*

Shaking cultures. Assays were performed on shaking cultures at a cell density of 2×10^6 cells/mL. Following drug treatments, the cultures were diluted as indicated, and 100-µL

samples were assayed as described above.

BacTiter-Glo (www.promega.com/pnotes/88/12162_02/12162_02.pdf) was designed for use with bacteria, and CellTiter-Glo (www.promega.com/pnotes/81/9939_02/9939_02.pdf) was designed to be used with mammalian cells. A detailed description of the basis of the assays and cited literature can be found at the above links. Most of the results presented were obtained with BacTiter-Glo. However, the results with BacTiter-Glo were compared with viability measurements using CellTiter-Glo. Although CellTiter-Glo results in lower RLUs (nearly 8-fold), the dilution curves with both reagents were identical (data not shown) and indicated that either reagent can be used. However, CellTiter-Glo had the advantage that the half-life of the signal is considerably longer than that of BacTiter-Glo (5 h versus 30 min, respectively), making it a more stable and reproducible system for the assays. The rapid decay of the luminescent signal with BacTiter-Glo makes it crucial to read all the samples at precisely the same time after adding the reagent.

When performing the assays in rich medium such as HL-5, it is important to be mindful that the medium contains ATP, and an appropriate background needs to be subtracted. When there is a lot of cell death (and a low number of remaining viable cells), the luminescence readings of these samples can be lower than the background luminescence of the medium alone. We have determined that this is not due to a change in pH of the tested samples, but rather is due to the reduction of the ATP in the medium, presumably by enzymes that are released from the lysed cells. To circumvent this, samples of the medium included for background measurements can be treated with alkaline phosphatase to remove the ATP from the medium (data not shown). We do not recommend exchanging the medium to a buffered salt solution before the assay, because we feel that the centrifugation and subsequent induction of starvation adds additional stress to the cells, which affects their metabolic state.

RESULTS AND DISCUSSION

Sensitivity and Range of the Assay

To determine the applicability of the commercially available luciferase-based cell viability assay for use in *D. discoideum*, we first determined the range of cells that can be assayed. Figure 1A shows a 2-fold dilution series of logarithmically growing *D. discoideum* cells (2×10^6 cells/mL) assayed with BacTiter-Glo. The assay gave a linear response between 100 and 25,000 cells/well, at which point the assay becomes saturated. Figure 1B shows the growth curves of cells cultured in 96-well plates in which the starting number of cells ranges from 100 to 800 cells. In all cases, there is a small lag-phase followed by growth, which reflects the initial cell number in the cultures. Taken together, it is clear that when applied to *D. discoideum*, this assay can accurately (*i*) measure cell number over a wide range of cell concentrations and (*ii*) monitor the growth of cultures.

Monitoring Survival After Drug Treatment

Cisplatin. *D. discoideum* has been used as a lead genetic model to study the underlying mechanisms of resistance to the widely used anticancer drug cisplatin [*cis*-dichlorodiamine platinum (II)] (3,4). Thus, this drug was chosen to evaluate how the luminescence assay works for measuring viability after drug treatment. Previous work on *D. discoideum* and human cells indicates that cisplatin does not have an immediate effect on cell viability, and cell death increases over time.

Identical 96-well plates were seeded with the indicated number of cells/well spanning the linear range of the assay, and multiple plates were treated with the same concentrations of cisplatin (150 or 300 µM final concentration). Each day, one plate was assayed for cell viability. Figure 2A shows the effect of cisplatin on viability as a function of the different initial cell densities over 3 days. In each case, the data are compared with a parallel sample that had not received the drug. After 1 day of incubation, it can be seen that both 150 and 300 µM cisplatin are cytotoxic and that 300 µM is more effective. The level of cytotoxicity at each drug concentration is essentially constant in wells containing between 100 and 6000 cells. At initial higher cell numbers, there appears to be a lower level of cytotoxicity, and this is almost certainly due to the untreated control cells having grown beyond the linear range of the assay (e.g., 12,500 cells grow beyond 25,000 cells in 24 h), therefore producing an erroneously low value for the untreated samples. After 2 days of incubation with the drug, there is increased cytotoxicity with both 150

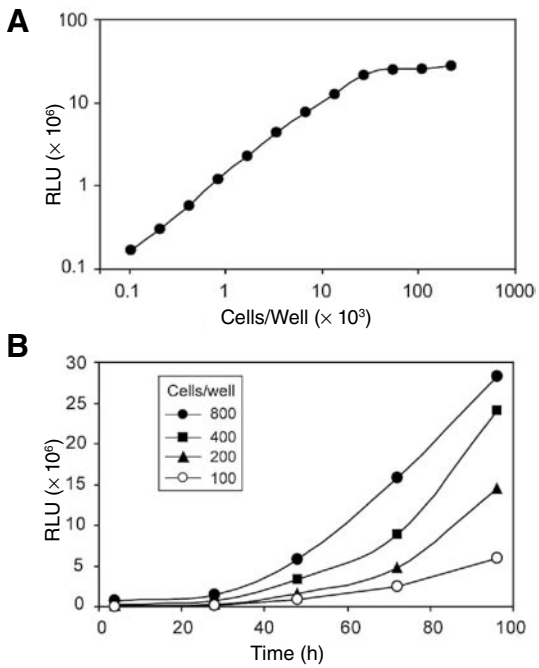

Figure 1. Determination of the linear range of the assay. (A) *Dictyostelium discoideum* Ax4 cells (at concentration of 100–200,000 cells/well in 100 µL) were plated in opaque 96-well plates and were reacted with 100 µL BacTiter-Glo for 30 min at room temperature. The data are the average of four replicates. (B) Cells were plated in different numbers of cells/well as in panel A and were allowed to grow for 6 days at 22°C. Each day, one plate was removed and developed as in panel A. The number of cells/well is indicated in the key. The data are the average of four replicates. Plotting the data on a log scale (not shown) shows that the growth is logarithmic. RLU, relative luminescence units.

and 300 µM cisplatin, and the cytotoxicity remains relatively constant over the same range of initial cell number. However, on day 3, the cells surviving treatment with 150 µM cisplatin have clearly begun to grow, and only the samples representing the lowest initial number of cells/well show a consistent level of cytotoxicity. The 300 µM samples show slightly more cytotoxicity than was seen on day 2. From these data, it is clear that the level of cytotoxicity observed at each day is a combination of the level of cell killing and the growth of the surviving cells.

These data support previous work showing that cisplatin does not have an immediate cytotoxic effect and takes time for the effect of the DNA lesions to be manifest. Moreover, the data reinforce the data in Figure 1, showing the need to work with cell numbers within the linear range of the assay. Overall, the data indicate that the assay is well suited to this kind of experiment and could be easily scaled up to assay many drugs by choosing one target cell number within the linear range of the assay and one or two time points.

It is reasonable to assume that these considerations apply to any type of cell and to any kind of assay and is almost certainly the cause of the some of the variability of results in drug studies reported in the literature. In assays in which time of incubation with the developing reagent is very sensitive [as with BacTiter-Glo or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays], this poses even more of a problem, because the control (untreated samples) exceed the linear range of the assay faster. However, it is clear that this assay can provide an accurate measurement of sensitivity to cisplatin when conditions are appropriately determined and controlled.

It has been shown that altering the levels of the enzymes sphingosine kinase or sphingosine-1-phosphate (S-1-P) lyase in *D. discoideum* cells alters the sensitivity to cisplatin. Increasing sphingosine kinase or lowering S-1-P lyase results in increased resistance to cisplatin, while either lowering sphingosine kinase or raising S-1-P lyase increases sensitivity to cisplatin. Pharmacologically lowering sphingosine kinase also increases sensitivity to cisplatin (7,8). Thus, it was important to determine if the luminescence assay could reproduce these results. To this end, we tested the sensitivity of spingosine kinase A (SgkA) overexpressing cells and sphingosine kinase A, B, and A/B null mutants to increasing concentrations of cisplatin. In the experiment shown in Figure 2B, a single target cell/well number was used (1000 cells/well). The results are the same as had been obtained earlier using a traditional plaque assay (7,8), in which SgkA overexpressing cells were more resistant to cisplatin and the null mutants were more sensitive to the drug, when compared with the parental wild-type cells.

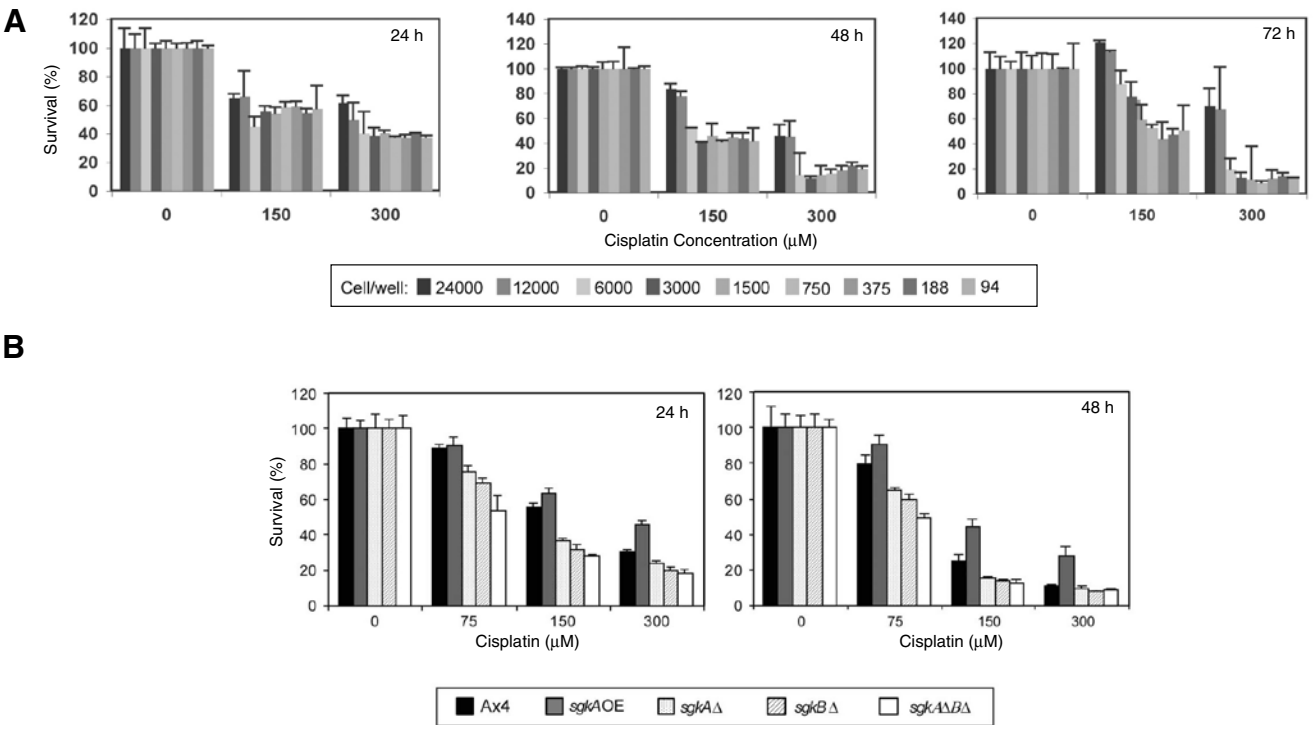

Figure 2. Sensitivity to cisplatin treatment. (A) *Dictyostelium discoideum* Ax4 cells. Identical plates were seeded with 2-fold serial dilution of cells, starting at 24,000 cells/well in a total volume of 90 µL/well at day 0, and were placed at 22°C. At 1 h, cisplatin was added at the indicated concentrations (0, 150, and 300 µM) in duplicate. Each day, one plate was removed, and viability was measured. The series of bars depicts the effect of cisplatin at different cells' densities over the first 3 days of the experiment. The number of cells/well is indicated in the key. The data are the average of duplicate determinations. The error bars represent the standard error. The values of the untreated samples are the simple average of the replicates. In the treated samples, the values represent the average ratio between the treated and the untreated samples. (B) Sphingosine kinase (*sgk*) mutants. Ax4 parental strain, as well as spingosine kinase A (SgkA) overexpressing (OE) cell, and *sgkA*, *sgkB*, and *sgkA/B* null mutants were assayed for cisplatin sensitivity as described in panel A at 24 and 48 h. One thousand cells/well were used for all strains. With the exception of one point (SgkA OE, 75 µM cisplatin, 24 h), all samples have $P < 0.04$.

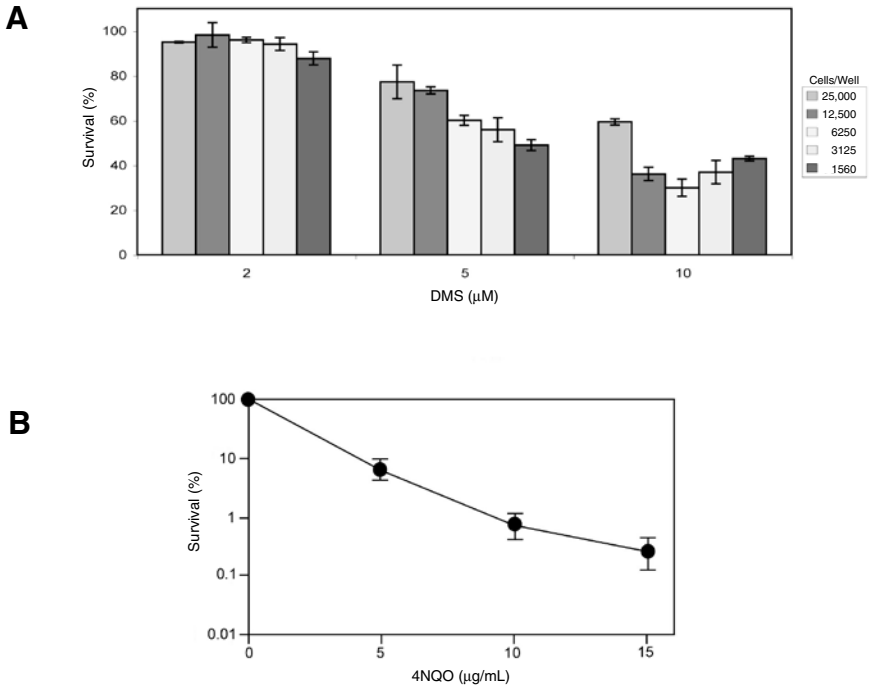

Figure 3. Sensitivity to dimethyl sphingosine (DMS) and 4-nitroquinone (4NQO). (A) DMS. A series of 2-fold dilutions of *Dictyostelium discoideum* Ax4 cells were plated in a 96-well plate, treated with 2, 5, and 10 µM DMS, incubated for 1 h, and assayed as above. The data are average of three replicates. (B) 4NQO. *D. discoideum* Ax4 cells at 2×10^6 cells/mL were treated with indicated concentrations of 4NQO for 24 h. Prior to the assay, the cells were diluted 1:50, and 100 µL each diluted culture were tested for viability. The data are the average of six replicates.

DMS and 4NQO. To determine the applicability to other drugs, the assay was used to measure survival after treatment with two other drugs. Both drugs have different mechanisms of cytotoxicity compared with cisplatin, which forms bulky adducts with DNA. DMS inhibits sphingosine kinase (8) and is toxic at certain concentrations in *D. discoideum*, and 4NQO is a γ irradiation mimetic (9). Both drugs require shorter exposure times to cause cytotoxicity. Despite these differences, the assay worked well in both situations. Figure 3A shows that at 10 µM DMS there is a constant level of cytotoxicity over the range of 1560–12,500 cells/well, similar to the cisplatin results in Figure 2. At 5 µM DMS, there is less cytotoxicity, and 2 µM DMS is essentially ineffective. Thus the data are similar to those obtained with cisplatin, although the assays were done over a much shorter period of time (1 h).

The results with 4NQO (Figure 3B), in which viability was monitored down to 0.5%, show that additional assay sensitivity can be achieved. The range of viability as described in the assay above (in 96-well plates) is limited to approximately 250-fold due to the maximum number of cells that can be innoculated per well, so as not to exceed the linear range of the assay. However, this range can be extended by performing the drug assay in shaking cultures at higher initial cell concentrations (e.g., 2×10^6 cells/mL) and then diluting the cultures before assaying them in the 96-well plates. This would allow the comparison of mutants that differ in drug sensitivity over several orders of magnitude, as was previously described for some *D. discoideum* mutants (10).

Comparison to Plating Assays

Based on the above results with different drugs and mutant strains, it is clear that the luciferase-based assay reflects what has been repeatedly seen by plating for viable cells. However there are differences, and it must be stressed that the unique properties of each drug be taken into account when the assay is used and when it is compared with the results of plating assays. For example, cells that are treated with 300 µM cisplatin for 3 h will show approximately 30%–40% cytotoxicity in viable plating assays (60%–70% survival), because the plates are scored 2–3 days after plating, which allows time for the cisplatin damage to be manifest and the cells to die. In contrast, the same cells will show almost 100% viability in the luminescence-based assay that is scored 3 h after the addition of the drug, because the cells have not died at this time and still contain ATP. Thus, in this case, longer incubation times of 1–2 days are needed for the luminescence-based assay, and lower initial cell numbers must be used so that the number of control cells remains within the linear range of the assay even after 2 days of incubation. With drugs like DMS and 4NQO that kill cells faster, the assay can be done with shorter incubation times (e.g., as little as 1 h with the DMS).

Future Applications

The luciferase-based assay can be used in at least two high-throughput applications: (*i*) for drug screens, in which many drugs at multiple concentrations can be screened for cytotoxicity in 96-well plates and (*ii*) for screening mutant libraries generated by insertional mutagenesis (11). Neither application can be done by traditional plating assays or by visual examination of microtiter wells for cell density. We expect this assay to be widely used for these applications, but it can also be used for convenient quantitative measurement of cell number and growth when, for example, several *D. discoideum* strains are being prepared for an experiment. Overall, this assay has many advantages and uses, but the analyses presented here indicate that attention to conditions and interpretation is necessary.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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