INTRODUCTION

The colony formation assay is the most widely used and probably the most relevant assay for testing the effects on cultured cells of treatments such as irradiation, chemotherapy, and hyperthermia. A disadvantage of this assay is that colony counting can be laborious and time-consuming, and that many dishes or flasks are required to define one dose-response curve. This has prompted several groups to develop more rapid assays for cell killing. One of the more successful has been the MTT assay which utilizes 96-well microtiter plates and measures absorbance per well after an enzyme-dependent color reaction to assess the number of surviving cells (1-6). This technique has the double advantages of a rapid readout of absorbance per well using enzyme-linked immunosorbent assay-based equipment, removing the need for colony counting, and the possibility of using multiple treatment groups per plate.

Most reports of the development of rapid cell survival assays have been primarily concerned with drug testing, although recently Carmichael and colleagues have assessed the MTT assay for radiosensitivity testing (4). In their study, however, cells were first irradiated and subsequently plated in the microtiter plates. For our purposes, we wished to be able to treat cells in different wells not only with different drug doses, but also with different radiation doses. This would eliminate the need for separate vessels for treatment, followed by the transfer of treated cells to multiwell plates, and would thus simplify the assay. The present report describes the development of a rapid cell survival assay for use in radiobiology studies in which different rows of wells in a 96-well plate can be irradiated with different doses. In addition, total cell numbers were assessed by quantitative DNA staining with a fluorescent dye, instead of measuring viable cells by the enzyme-dependent reaction used in the MTT assay.

MATERIALS AND METHODS

Cell Lines. Two cell lines were used in this study; RIF1 mouse sarcoma cells and CHO. The RIF1 line is a radiation induced tumor cell line maintained by alternating in vitro and in vivo passages (7). Both lines were grown as monolayers in Ham's F10-medium plus 10% fetal calf serum. Doubling times for RIF1 and CHO cells were approximately 13 and 10 h, respectively, with plating efficiencies between 50 and 80%.

Irradiations. Cells were irradiated using an X-ray set operating at 200 kV and 20 mA with 0.5 mm Cu filtration. The dose rate at the cells was 1.09 Gy/min at a focus-cell distance of 43 cm. For irradiation of the 96-well microtiter plates, a special irradiation box was designed and built which allowed a vertical X-ray beam. The block has a metal handle which protrudes through a hole on one side of the box and contains 12 grooves spaced exactly the same distance apart as the distance between the centers of the 12 rows of wells on the multiwell plate. By inserting or withdrawing the block by means of this grooved handle, the block/multiwell plate could be moved one groove at a time (i.e., one row of wells) with respect to the X-ray beam. The underside of the 1.0-cm thick lead cover was 1.5 cm above the cells. Four separate lead covers were built, each containing a different shaped hole allowing between four and 16 wells (1/2 row up to two rows of wells) to be irradiated at one time (Fig. 1). Most irradiations were carried out using an irradiation field covering one row of eight wells (8 x 71 mm). The supporting block contained a hole immediately under the irradiated wells to minimize back scatter into adjacent rows. Thermoluminescent dosimetry measurements indicated that the two protected rows on either side of the irradiated row received approximately 3.5% of the dose given to the unprotected row. This was also checked by cell survival studies (see "Results").

Drug Treatments. Drugs were made up at 10 times the required final concentration in PBS and 20 μl was added to 200-μl growth medium per well (well area, 0.3 cm²). Cis-diaminedichloroplatinum(II) (cisplatin) was obtained as a 0.5 mg/ml solution (Platinol; Bristol Myers Co.); iododeoxyuridine and cysteamine were obtained as a powder from Sigma (Dorset, England). After the appropriate treatment time the cells were washed twice carefully with warmed PBS after first aspirating off the drug containing medium.

QF Assay. Between 3 x 10³ and 5 x 10³ cells in 0.2 ml were plated per well and treated with drug or radiation 1 day later. 3 days after treatment the cells were fed by replacing the overlying medium and 1 day later (5 days after plating) the cells were stained and counted.

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IUDR, 5-ido-2'-deoxyuridine; PLDR, potentially lethal damage repair, CHO, Chinese hamster ovary fibroblast(s); QF, quick fluorescence assay; PBS, phosphate buffered saline.
(100 μg/ml in 70% ethanol, 0.1 ml per well) was then added using a repeater pipet and left on the cells at room temperature for 30 min. The stain was then poured off, the cells washed once with PBS and the plates counted for fluorescence with no liquid overlying the cells. Tests with H33258 alone (no cells) showed that one wash with PBS was sufficient to reduce fluorescence to background levels. The plates were analyzed using a Microfluor TM reader which gave a printout of fluorescence per well for 96-wells within 30 s (Dynatech Laboratories Inc., Billinthurst, England). Excitation wavelength of the reader was 365 nm with an emission filter collecting fluorescent light in the band 445–455 nm. Surviving fractions (SF) were calculated according to:

$$ SF = \frac{F_T - B}{F_U - B} $$

where $F_T$ and $F_U$ are fluorescence values of treated and untreated cells respectively and $B$ is the background fluorescence. For the best agreement between the fluorescence and colony assays, it was necessary to use the fluorescence of cells treated with a supralethal dose of the agent under test as the background ($B$) value (see “Discussion”). Several makes of microtiter plate were tested, from which Falcon was selected as having the lowest natural fluorescence in the absence of cells or H33258.

RESULTS

Staining Tests. The linearity of the microfluor reader was tested with a series of H33258 concentrations (no cells) and a linear relationship between fluorescence and stain concentration was found; fluorescence per well values varied from 25 (background fluorescence of empty plates) to approximately 3000 (arbitrary units). To test for linearity between cell number and fluorescence, a range of cell numbers per well were plated, allowed 6 h for attachment and stained with H33258 as described earlier (see “Materials and Methods”). A good linear relationship was found between the number of cells per well and fluorescence (Fig. 2). The maximum fluorescence value for a confluent monolayer of cells was found in separate experiments to be around 600, equivalent to approximately $2 \times 10^5$ cells per well. (The data shown in Fig. 2 and Table 1 are for nonconfluent cells.)

Because only one-fifth of the machine’s capacity was being used ($600 \text{ versus } 3000$), attempts were made to increase the fluorescence per cell. It was found, however, that increasing the stain concentration up to 600 μg/ml had no effect on fluorescence (Table 1, Experiment B) and that increasing the staining time up to 24 h was also ineffective (data not shown). Other DNA stains excitable under UV illumination, i.e., 4,6-diamidino-2-phenyl indol and H33342, were also tested but found to be worse or no better than H33258 with regard to fluorescence per cell. Finally, attempts were made to bring the cells and/or DNA into solution, since the reader was designed to optimally measure fluorescence in solution. Triton X-100, water, trypsin, and lysis medium (high pH plus sodium dodecyl sulfate) were therefore, separately, tested. Lysis medium increased both cell related fluorescence and background fluorescence, resulting in no net gain of sensitivity. Triton X-100 gave similar results to lysis medium, and water and trypsin were ineffective. The fluorescence range therefore remained approximately 30–600.

![Fig. 1. Diagram of microtiter plate irradiation system. The 96-well plate is placed on a holder which is movable with respect to the X-ray beam. The field size is determined by the hole in the lead cover. Four covers with different hole sizes were manufactured.](image)

![Fig. 2. Linearity of fluorescence with cell number. Varying numbers of RIF1 cells were plated and stained 4 h later with H33258 (600 μg/ml). Errors are ± 1 SEM; eight wells per group.](image)

Table 1 Optimal stain concentrations

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cellsa</th>
<th>Stain concentrationb</th>
<th>Fluorescence per wellc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>–</td>
<td>100</td>
<td>36.5 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10</td>
<td>74.3 ± 10.8</td>
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<tr>
<td></td>
<td>+</td>
<td>50</td>
<td>171.3 ± 25.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>100</td>
<td>209.3 ± 26.8</td>
</tr>
<tr>
<td>B</td>
<td>–</td>
<td>0</td>
<td>28.1 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>600</td>
<td>29.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>34.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>100</td>
<td>81.7 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>200</td>
<td>89.4 ± 13.8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>400</td>
<td>78.8 ± 10.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>600</td>
<td>75.0 ± 11.9</td>
</tr>
</tbody>
</table>

*a* RIF1 cells plated per well: $10^5$ exp. A; $3 \times 10^4$ exp. B.  
*b* H33258, μg/ml in 70% ethanol, 30 min.  
*c* Mean ± 1 standard deviation; n = 7–18.
which was sufficient to define survival curves over a range between 1 and 2 decades of cell killing (see below).

**Drug Experiments.** RIF1 cells were treated with various concentrations of the cytotoxic agent cisplatin for an exposure period of 1 h and tested for the fraction of surviving cells using both the QF and colony assays. There was good agreement between the assays over a 2-decade range of cell survival (Fig. 3). Good agreement was also found for cytotoxicity of IUdR and cysteamine (data not shown).

**Radiation Experiments.** The results of an experiment with RIF1 cells in which each alternate row of eight wells was irradiated using a graded series of X-ray doses is shown in Fig. 4. Increasing doses produced increasing killing as assessed by the fluorescence measurements. The control (unirradiated) points are plotted at their respective positions on the multwell plate, i.e., the furthest right hand point was derived from the row lying between rows receiving 8 and 10 Gy. These cells should have received 0.6 Gy scattered radiation (3.5% of 18 Gy) based on the thermoluminescent dosimetry measurements. Considering the steepness of the curve for irradiated cells in this region, it is possible that 0.6 Gy may just have been detected by a decrease in fluorescence. The lack of an observable decrease using this biological dosimetry test indicated an upper limit of 3.5% scattered irradiation to adjacent rows. It further indicated that, if desired, adjacent rows could be irradiated with different doses as part of a dose-response curve with little fear of dose perturbations caused by scattered irradiation.

The flattening of the X-ray curve at high doses (Fig. 4, top) is due to approaching the background fluorescence of lethally irradiated cells. The highest dose used, 15 Gy, is expected to give a survival of approximately 1 in 10⁵ (8) and since only 3 × 10³ cells were plated per well, the vast majority were lethally irradiated. These doomed cells increase in number by a factor between 1.5 and 2 in the 4 days after irradiation (separate experiments; data not shown) giving rise to a background fluorescence almost twice that of an empty well. If the 15 Gy value was used for the background subtraction, the survival curve shown in Fig. 4, bottom, resulted. This was close to that determined by the colony assay from three experiments carried out over the same period (hatched area, Fig. 4, bottom).

The ability of the assay to detect sublethal damage repair after irradiation is shown in Fig. 5. The 2-fraction curve lay to the right of the single-dose curve, indicating significant sublethal damage repair in this RIF1 cell line, consistent with the curvature on the single-dose curve and with previous repair experiments using the colony assay (9). Repair of PLDR occurring after single doses of radiation could not be demonstrated using this assay since no trypsinization was carried out after treatment. Attempts were made to stimulate PLDR by incubating in low serum for several hours after treatment but no repair of this type of damage could be demonstrated.

**Radiomodifier Experiments.** The ability of the thymidine analogue IUdR to sensitize CHO cells to radiation is shown in Fig. 6. Both QF and colony formation assays demonstrated that cells incubated for 18 h with 3 μM IUdR prior to irradiation were significantly more sensitive to X-rays. The curves for the two assays lay close to each other although the shapes for IUdR-treated cells were somewhat different. Mean enhancement ratios taken from three survival levels over the range tested were 2.4 and 1.9 for the QF and colony assays, respec-
A RAPID RADIOSENSITIVITY ASSAY

1.0

X-RAY DOSE (GY)

Fig. 6. Radiosensitization of CHO cells by IUDR (3 μM; 18 h): comparison of QF (solid lines) and colony assays (broken lines). Curves have been corrected for killing by drug alone.

1.0

X-ray dose (Gy)

Fig. 7. Radiosensitization of RIF1 cells by cisplatin given as a 1-h exposure immediately before X-rays, as measured by the QF assay. The drug doses in μM are indicated against the curves which have been corrected for killing by drug alone.

1.0

Surviving fraction

X-ray dose (Gy)

Fig. 8. Measurement of radioprotection of RIF1 cells by cysteamine using the QF assay. Drug doses are indicated against the curves which have been corrected for killing by drug alone.

DISCUSSION

The rapid assay described here utilizes 96-well microtiter plates and uses the ratio of cell numbers, measured by DNA fluorescence, as a measure of cell survival. It is rapid not so much in the overall growth time, but in the number of different treatment groups possible on one plate and in the rapidity of the fluorescence readout from the automated microtiter plate reader (30 s for 96 wells). The assay is applicable to any cell line which grows as an attached monolayer. In addition to the two rodent cell lines used here, we have also successfully used the assay for chemosensitivity testing of two human cell lines. Although not tested, it is presumed that the assay would be less suitable for cells which grow in suspension, due to difficulties with the necessary washing steps during treatment and staining.

The assay depends on the growth of untreated cells. If the cell number ratio is assessed too late, the control cells will have reached confluence and ceased to increase in cell number. In this case, killing will be underestimated. The optimum assay time is just before the untreated cells stop growing exponentially and when the survivors of the treated populations have outgrown the number of doomed cells, i.e., when regrowth in the treated populations is seen. Assay conditions (cell numbers plated, time of assay) need to be optimized for each new cell line. Growth curves for fluorescence can easily be measured and inspected for the moment of departure from exponential growth. For RIF1 and CHO cells it was found that feeding on Day 4, 1 day before staining, was necessary to keep cells in exponential growth long enough to obtain adequate fluorescence for survival measurements. It was also found that some cells were lost during washing after drug treatment. A higher cell number was therefore usually plated (5 × 10³ instead of 3 × 10³) for treatments involving drugs.

Fig. 9 illustrates the effect of choice of assay time and background subtraction for a series of hypothetical growth and regrowth curves. Surviving fractions after 2, 4, and 7 Gy were assumed to be 0.5, 0.1, and 0.01, respectively (Fig. 9A), the growth of surviving cells were assumed to parallel control growth and the doomed cells were assumed, on average, to increase by a factor of 2, a value close to that found in practice for RIF1 cells. Cell number ratios after different growth times (Fig. 9B) show that curves assessed at early times approach the "true" curve but still contain an apparent resistant tail. This is due to the high contribution of doomed cells at high doses. If the doomed cell number is estimated from a dose at which effectively all cells are doomed (e.g., 15 Gy) and this number subtracted from both treated and control values, the curves...
In the present report, the development is described of a system for irradiating individual rows or part rows of wells, facilitating the use of the assay in radiobiological tests. The irradiation system could equally well be used with the MTT and fluorescence assays. One disadvantage of this irradiation system is that irradiations under hypoxic conditions are not possible due to the oxygen diffusion properties of the plastic in the microtiter plates. For hypoxia studies, cells must therefore be first irradiated in suitable vessels and subsequently transferred to the microtiter plates for growth and survival measurements, as was reported by Carmichael and colleagues (4).

In conclusion, the assay described here, together with the accompanying irradiation system, is suitable for both chemotherapy and radiotherapy studies, including those of radiosensitization by sensitizers or protectors. By appropriate choice of assay conditions, survival values similar to those from the clonogenic assay can be obtained. Adherence to plastic is a requirement of the assay and optimized assay conditions may be different for each cell line. It was designed specifically for use with established lines in culture, although it is conceivable that in the future it could be adapted for the study of fresh human biopsy material for predictive assay purposes in a manner similar to that described by Brock and colleagues (12).

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REFERENCES


Fig. 9. Simulation of a growth assay for radiation treatments. A, growth curves of total cells (solid lines), surviving cells (broken lines), and doomed cells (15-Gy curve) for three radiation doses. All curves plateau when cells become confluent (2 x 10^5 cells per well). B, survival curves derived from A using the ratio of treated to control cells (total) as a measure of surviving fraction and assessed at Days 3, 4, or 5 after plating. C, survival curves derived from A but using the 15-Gy value for background subtraction.

The assay described here is similar to the MTT assay in that they both employ 96-well microtiter plates and both are essentially growth assays (3, 10). They also cover approximately the same range of cell survival (1-2 decades). The fluorescence assay has the advantage of a shorter staining time (30 min), compared with the overnight dimethyl sulfoxide step sometimes employed in the MTT assay (3). It is also free of metabolic effects that can influence the MTT assay (5). The MTT assay may have an advantage, however, for treatments producing rapid metabolic shut down and death, in which effects could be measured without the need to wait for growth of untreated cells. A fluorescence-based microtiter plate assay has also been described by Sacki et al. (11) but in contrast to the present assay and similar to the MTT assay it depends on intact cell metabolism. It will therefore have the same advantages and disadvantages at the MTT assay. No radiation experiments have been described with this assay.
Rapid Fluorescence-based Assay for Radiosensitivity and Chemosensitivity Testing in Mammalian Cells *in Vitro*

Adrian C. Begg and Els Mooren


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