Clonogenic assay of cells *in vitro*

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Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. Clonogenic assay is the method of choice to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents. Only a fraction of seeded cells retains the capacity to produce colonies. Before or after treatment, cells are seeded out in appropriate dilutions to form colonies in 1–3 weeks. Colonies are fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v) and counted using a stereomicroscope. A method for the analysis of radiation dose–survival curves is included.

INTRODUCTION

In 1956, Puck and Marcus[1] published a seminal paper describing a cell culture technique for assessment of the clone- or colony-forming ability of single mammalian cells plated in culture dishes with a suitable medium. The selected medium was supplemented with a large number of heavily irradiated "feeder" cells, which served to condition the microenvironment of the much smaller number of cells to be tested for their clone formation. The authors carried out experiments that yielded the first radiation–dose survival curve for HeLa cells in culture irradiated with X-rays. They showed that these mammalian cells were much more radiosensitive than assumed earlier for cells in tissues, with mean lethal doses in the range of 1–2 Gy.

This clonogenic assay has been used in the ensuing decades for a large variety of studies with many types of cells, using improved complex culture media, which have partly eliminated the need for feeder cells. The assays detect all cells that have retained the capacity for producing a large number of progeny after treatments that can cause cell reproductive death as a result of damage to chromosomes, apoptosis, etc.[2].

Clonogenic assays have also been developed for stem cells in various tissues by *in vivo* techniques[3]. In 1961 (ref. 4), stem cells from the bone marrow of mice were shown to produce colonies reproducibly in spleens of heavily irradiated recipient animals.[4] Later, skin stem cells were shown to be capable of producing nodules in the irradiated skin of mice after doses that impair proliferation of the majority of cells in a selected small area. Furthermore, stem cells in the crypts of mouse jejunum were shown to produce colonies in heavily irradiated regions of the intestine. Although the ranges of doses that can be studied with these *in vivo* systems are subject to limitations, the results obtained for sensitivities to X-rays are in good agreement with data for cells in culture[3].

In addition to methods for cells in culture and cells in normal tissues, clonogenic assays have been developed for cells from tumors in animals[5]. Cells in transplantable tumors are harvested to yield cell suspensions and known numbers of cells are injected into recipient animals where they can develop into new tumors. If donor tumors are irradiated before harvesting, a fraction of the cells will lose clonogenic capacity and, as a consequence, larger numbers of tumor cells are required to yield tumors in recipient animals. From a few transplantable tumors in rats and mice, harvested cells can be cultured directly and quantitatively *in vitro* to produce clones and provide information on the effectiveness of treatments *in vivo*.

Many studies performed with all these systems have yielded information about differences in sensitivity to radiation and chemotherapeutic agents among tumors and normal tissues and about modification of treatment effectiveness by various conditions and modes of application. The capacity for continued proliferation of stem cells in tissues is a prerequisite for the continued integrity and function of normal tissues, whereas in tumors eradication of the capacity for unlimited proliferation of all stem cells is required for the prevention of recurrences.

MATERIALS

REAGENTS

- Appropriate culture medium containing serum
- Phosphate-buffered saline (PBS) pH 7.2
- Trypsin (Invitrogen; trypsin–EDTA (0.5% trypsin with EDTA–4Na; Invitrogen, cat. no. 15400-054) 10×). The trypsin solution is freshly prepared before the experiment from a stock solution containing 0.5 g l^{-1} trypsin, 0.2 g l^{-1} EDTA and 0.85 g l^{-1} NaCl
- Isotonic buffer for cell counting when using a Coulter counter
- Colony fixation-staining solution, glutaraldehyde 6.0% (vol/vol), crystal violet 0.5% (wt/vol) in H_2O

EQUIPMENT

- Pipettes
- Culture dishes or six-well plates
- Tubes for dilution
- Coulter counter or hemocytometer
- Stereomicroscope
- Colony counting pen
- Statistical analysis software (SPSS)

PROCEDURE

Initial handling of cells

1| Before any experiment, ensure that all required materials from sterile pipettes, sterile test tubes, culture dishes and six-well plates are at hand. Warm the medium, PBS and trypsin to 37 °C. Work out the cell dilutions and label the dishes or plates. The experiment has to continue smoothly to limit the total time, preventing adverse effects of pH and temperature changes.

2| Harvesting cells from a donor culture is performed using trypsinization. As described in Steps 2–6, to detach cells from the plastic, the overlying medium is removed and cells are washed with PBS. PBS is removed and replaced by a solution containing trypsin.

3| Remove the medium above the cells.

4| Wash the cells with PBS.

5| Trypsinize cells to produce a single-cell suspension. The trypsin solution should be left over the cells until they round up; this may be inspected under the microscope.

6| When cells start to round up indicating detachment from the culture dishes, resuspend the cells in medium to inhibit trypsinization. Adding sufficient volume of medium (more than 3× the volume of trypsin) supplemented with serum neutralizes the trypsin solution. Detach the cells by pipetting up and down the medium with the cells.

7| Count the cells.
▲ **CRITICAL STEP** The accurate number of cells that are plated is required to obtain the correct data for plating efficiency (PE) for unirradiated controls or after a certain treatment for a proper survival calculation.

8| Dilute the cell suspension into the desired seeding concentration and seed into flasks or plates as desired (see the next step for information on how many to seed).
▲ **CRITICAL STEP** The dilutions have to be performed accurately to seed the correct number of cells.

Clonogenic assay setup

9| There are two essentially different ways to perform studies using this assay: In option (A), cells are plated before treatment. Cells are harvested from a stock culture and plated at appropriate dilutions into (cluster) dishes. After attachment of the cells to the dishes, which generally takes 2 h or more, the cells are treated. The treatment has to be performed before cells start replicating; otherwise, the numbers of cells per dish will increase, yielding more colonies. After treatment, the dishes are placed in an incubator and left there for a time equivalent to at least six potential cell divisions. This method is often used for a quick screening of the sensitivity of cells to different treatments. In option (B), cells are treated in dishes and subsequently re-plated in appropriate dilutions to assess clonogenic ability. The replating may be performed immediately after treatment (IP) or it may be delayed (DP) to allow repair processes. This method is used especially in radiobiological research to determine potentially lethal- and sublethal damage repair.

(A) Plating before treatment

(i) Harvest exponentially growing cells and re-plate an appropriate number of cells per dish or per well of a cluster dish (this depends on the severity of the treatment; if you do not know the appropriate effect, use different dilutions of different cell numbers). Allow time for the cells to attach to the plastic. Usually, this takes a few hours at 37 °C. Check attachment of cells by using a microscope.

(ii) Treat the cells in the dishes or wells as necessary in your experiment and place the dishes thereafter, at least in duplicate, in an incubator. The atmosphere in the incubator has to be adapted to the requirements of the growth medium of the cells used, for example, excess CO_2 content. Never forget the humidity: place a tray with clean water at the bottom in the incubator to prevent drying up of the culture medium in the dishes during incubation. Leave the dishes in the incubator until cells in control dishes have formed sufficiently large clones (clones are considered to represent viable cells if they contain in excess of 50 cells, that is the minimum to be counted).

(B) Plating after treatment (IP or DP)

(i) Harvest cells after treatment. Count the number of cells in the resulting cell suspension using a Coulter counter, and dilute in sterile tubes so that 100 or up to 10^4 cells after severe treatment can be pipetted into the test wells. To study potentially lethal damage repair after ionizing radiation (the difference in survival between IP and DP: DP cells usually show a higher survival than IP cells), cells are re-plated immediately or delayed after treatment. When it is not possible to process IP cells directly after treatment, it is best to keep the cells on ice until handling. The delay time for delayed plating is usually between 6 and 24 h. After 6 h, potentially lethal damage repair is complete[6]. If you do not know the severity of the treatment, use different dilutions. Pipette the cells in the test dishes and at least in duplicate.

(ii) Place the dishes in an incubator and leave them there until cells in control dishes have formed sufficiently large clones.

Fixation and staining of colonies

10| Remove the medium above the cells.

11| Rinse carefully with PBS.

12| Remove the PBS and add 2–3 ml of a mixture of 6.0% glutaraldehyde and 0.5% crystal violet.

13| Leave this for at least 30 min.

14| Remove the glutaraldehyde crystal violet mixture carefully and rinse with tap water. Do not place the dishes or plates under the running tap, but fill the sink with water and immerse the dishes or plates carefully.

15| Leave the dishes or plates with colonies to dry in normal air at room temperature (20 °C).

■ **PAUSE POINT** Colonies can be counted up to at least 50 weeks after staining.

Counting the colonies

16| The standard procedure is to count using a stereomicroscope and an automatic counting "colony counter pen." Determine the PE of control cells, that is, the fraction of colonies from cells not exposed to the treatment. In every experiment, the PE must be determined, as small changes in conditions may influence this factor. The surviving fraction of cells after any treatment is always calculated taking into account the PE of control cells . In **Figure 1a,** a typical example of the result of a clonogenic assay of SW-1573 human lung tumor cells is presented.

● **TIMING**

This is estimated for an assay with five radiation doses: 0, 2, 4, 6 and 8 Gy
Steps 1–9: 1–2 h
Steps 10–15: 1 h
Step 16: About 10 min per six-well plate

? TROUBLESHOOTING

When cells are plated at low densities, they sometimes do not form proper colonies and PE may drop below 10%. Continuous cell lines rarely have this problem. However for primary cultures, the PE may drop even below 0.5% or even zero. Some alternative methods are available[7]: adding conditioned medium to the cells, growing them on feeder layers or growing them by the soft agar method.

Conditioned medium

Conditioned medium is the medium from a growing culture and it contains all kinds of growth factors produced by dividing cells. By adding this medium to colony-forming cells, the cells are stimulated to divide and form colonies.

Feeder layer

A feeder layer consists of a cell culture, usually of fibroblasts, that is irradiated with 30–40 Gy. The irradiated cells are sterilized and do not divide anymore, but still produce growth-stimulating factors. The colony-forming cells are seeded on the feeder layer.

Soft agar method

Some cells do not form colonies or very dispersed colonies. For these cells, the soft agar method might be useful. An agar suspension (0.3% agar) containing colony-forming cells is plated over an agar underlay (2.0% agar). The agar will hold the colony together. Avoid the hyperthermia effect and ensure that the temperature of the agar is not too high. Agar is a gel at 36.5 °C but is liquid at 45 °C.

ANTICIPATED RESULTS

Combinations of radiation with other treatment modalities

Surviving fractions as a function of dose can also be determined by using the clonogenic assay when combination treatments are carried out, for example, drug–ionizing radiation interactions. However, the drugs might influence the proliferation rate. Then, colony formation might take a longer time period than for radiation alone. The surviving fraction of a "drug-only" control should be determined as well. The radiation dose–survival curve may be modified by the additional treatment, yielding results expressed as a dose-modifying factor, dose enhancement ratio or sensitizer enhancement ratio. In case of hyperthermia, the term thermal enhancement ratio is used. A procedure using the computer program SPSS to test statistically for a significant change of the control and modified radiation dose–survival curve is incorporated at the end of this paper.

When for a certain drug a dose-modifying factor is calculated, the ratio of the radiation dose at a certain survival level after radiation alone to that for the combined treatment should then be calculated and not the ratio of the surviving fractions at a certain radiation dose.

TABLE 1 | Different cell lines of human and Chinese hamster origin, culture medium (all from Invitrogen) in which they grow, percentage of CO_2 of the incubator and PE.

Cell line	Cell type	Human/animal	Medium	CO_2 (%)	PE (%)
SW1573	Lung carcinoma	Human	L-15	0	50–80
Gli-6	Glioblastoma	Human	D-MEM	10	50–80
RKO	Colorectal	Human	McCoys 5A	5	70–90
DLD1	Carcinoma	Human	RPMI	5	40–60
AG1522	Fibroblast	Human	D-MEM/F12	5	10
PC3	Prostate tumor	Human	RPMI	5	50–60
DU-145	Prostate tumor	Human	RPMI	5	50–60
V-79	Lung fibroblast	Chinese hamster	MEM	5	70–100
CHO	Ovary	Chinese hamster	MEM	5	70–100

To the medium of AG1533, 15% serum should be added. All cell lines grow in medium with 10% serum. Abbreviation: PE, plating efficiency.

Plating efficiency and surviving fraction

Different cell lines have different plating efficiencies. In **Table 1,** some examples of plating efficiencies of various cell lines of human or animal origin with the appropriate culture medium are listed.

When untreated cells are plated as a single-cell suspension at low densities of 2–50 cells cm^{-12}, they will grow to colonies. PE is the ratio of the number of colonies to the number of cells seeded:

$$\mathrm{PE} = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} \times 100\%$$

The PE of the example in **Figure 1** is

$$\frac{(70/100+115/200)}{2} = 0.64\,(64\%)$$

The number of colonies that arise after treatment of cells, expressed in terms of PE, is called the surviving fraction (SF):

$$\mathrm{SF} = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded} \times \mathrm{PE}}$$

The surviving fraction after 4 Gy of the example in **Figure 1** is

$$\frac{(39/400)+(66/800)}{2\times 0.64} = 0.14$$

Analysis of the radiation dose survival curves

Survival (S) data after a radiation dose (D) (**Fig. 2**) are fit by a weighted, stratified, linear regression according to the linear–quadratic formula $S(D)/S(0) = \exp(\alpha D+\beta D^2)$[8–11]. To be able to determine the linear parameter α and the quadratic parameter β, an SPSS datafile is created for each radiation dose–survival curve separately (e.g., without and with pretreatment) with the following variables: number of the experiment (all data must be derived from at least three separate

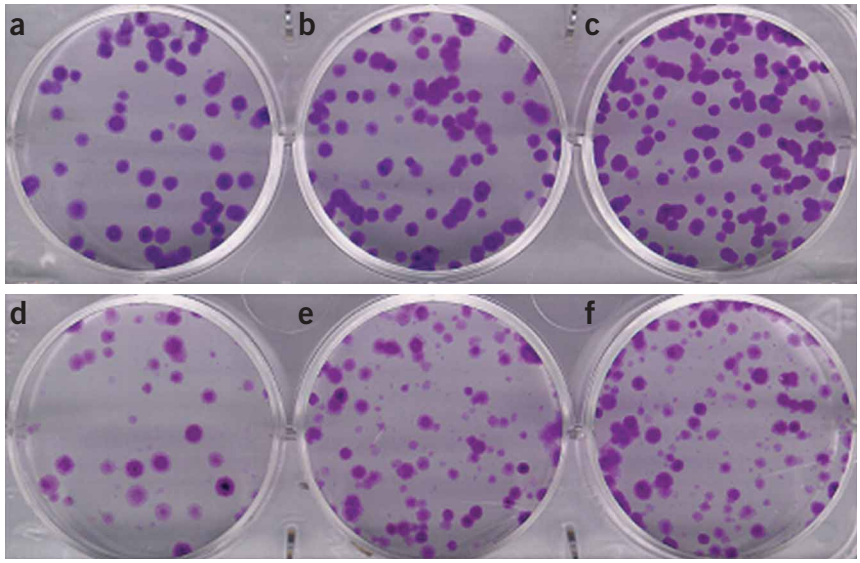

Figure 1 | Clonogenic assay performed in six-well plates, with clones produced by SW-1573 lung tumor cells. (**a**,**b**) Untreated controls with 70 and 115 clones, respectively, formed after seeding 100 and 200 cells. (**c**) Here too many clones are overlapping after seeding 400 cells and therefore this well is not reliable for counting. (**d**,**e**) After 4 Gy radiation treatment, 39 and 66 clones are formed, after seeding 400 and 800 cells, respectively. (**f**) Here too many colonies are overlapping after seeding 1,600 cells and therefore this well is not countable. After 4 Gy, many cells still divide 2–3 times and small colonies of less than 50 cells are formed but not scored for survival.

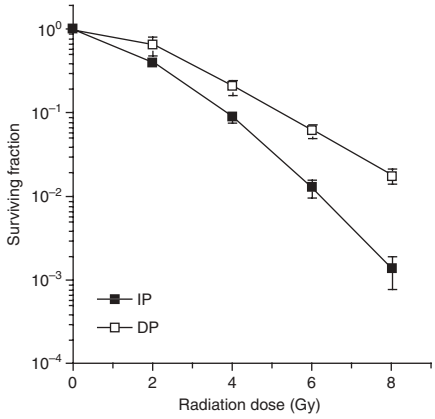

Figure 2 | Survival curves of *Glioblastoma multiforma* cells plated immediately (closed symbols) and delayed plated (open symbols) after irradiation. The survival curves derived from clonogenic assay experiments are significantly different ($P<0.01$) as tested with the above-described analysis[10] (see ref. 9).

experiments: e.g., 0, 1 and 2), "dose" (e.g., 1, 2, 4, 6 and 8 Gy), number of cells plated ("cells", e.g., 800 cells for 4 Gy), number of surviving colonies ("colonies", e.g., 66 colonies after 4 Gy) and PE of that experiment (e.g., 0.64 for all doses of experiment 1). Then, the following transformations are performed (under transform/compute):

For the quadratic term	"D2" = dose*dose
For survival	"S" = In (colonies/cells) − In (PE)
For the weight of the colonies found	"W" = colonies*cells/(cells − colonies)

Subsequently, a linear regression (under analyze/regression/linear) is performed with "*S*" as a dependent variable, "dose" and "D2" as independent variables and "W" under WLS. Confidence intervals and R^2 change are included in the regression (under statistics). No constant is included in the equation (under options), because the regression passes through the origin [1,0]. The model summary of the output of the regression states the *P*-value for the hypothesis that there is no relationship between radiation dose and survival (for $P<0.05$, there is a relationship). The coefficients of the output provide linear and quadratic parameters with standard error and confidence intervals. These parameters can be used to calculate radio enhancement ratios at 10% survival.

To test statistically the difference between two different curves (**Fig. 2**)[7], a new SPSS datafile was created with the first five variables of both curves. Two variables were created to separate the two curves, for example, "control" for the radiation-only curve and "treated" for the pretreated radiation curve. The data of the radiation-only curve were marked "1" in the column of the "control" variable and "0" in the column of the "treatment" variable. The data of the pretreated curve were marked in reverse. In addition to the three transformations described above, four more variables were transformed:

"controldose"	=	control*dose
"controlD2"	=	control*D2
"treatdose"	=	treat*dose
"treat2"	=	treat*D2

For the linear regression, an additional block for independent variables was created in which "dose", "D2", "controldose" and "controlD2" were inserted. After running the regression as described above, the model summary for model 1 again states the *P*-value for the hypothesis that there is no relationship between radiation dose and survival. The model summary for model 2 states the *P* value for the hypothesis that the data scatter was described best with one curve (for $P<0.05$, the data scatter is best described with two curves, i.e., a statistical difference between the pretreated curve and the control curve).

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COMPETING INTERESTS STATEMENTS The authors declare that they have no competing financial interests.

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