nature protocols

PROTOCOL EXTENSION

https://doi.org/10.1038/s41596-021-00615-0

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Analysis of clonogenic growth in vitro

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The clonogenic assay measures the capacity of single cells to form colonies in vitro. It is widely used to identify and quantify self-renewing mammalian cells derived from in vitro cultures as well as from ex vivo tissue preparations of different origins. Varying research questions and the heterogeneous growth requirements of individual cell model systems led to the development of several assay principles and formats that differ with regard to their conceptual setup, 2D or 3D culture conditions, optional cytotoxic treatments and subsequent mathematical analysis. The protocol presented here is based on the initial clonogenic assay protocol as developed by Puck and Marcus more than 60 years ago. It updates and extends the previous *Nature Protocols* article by Franken et al. in 2006. It discusses different strategies and principles to analyze clonogenic growth in vitro and presents the clonogenic assay in a modular protocol framework enabling a diversity of formats and measures to optimize determination of clonogenic growth parameters. We put particular focus on the phenomenon of cellular cooperation and consideration of how this can affect the mathematical analysis of survival data. This protocol is applicable to any mammalian cell model system from which single-cell suspensions can be prepared and which contains at least a small fraction of cells with self-renewing capacity in vitro. Depending on the cell system used, the entire procedure takes ~2–10 weeks, with a total hands-on time of <20 h per biological replicate.

This protocol is an extension to: *Nat. Protoc.* 1, 2315–2319 (2006) https://doi.org/10.1038/nprot.2006.339

Introduction

Since its development in the mid-1950s, the clonogenic assay has been used by numerous researchers to measure the self-renewing capacity of various mammalian cell model systems in vitro[1–4]. In this context, 'clonogenic' growth is conventionally defined as a cluster of ≥50 cells originating from one single cell. Initially used by Puck and Marcus to test the cytotoxic effects of ionizing radiation, it has proven a versatile and easy-to-handle technique of basic mammalian cell culture in a wide range of disciplines, including medical oncology, stem cell research, cell biology, pharmacology and toxicology[5–15]. Over years of extensive use, the protocol, which was originally designed to assess the self-renewing capacity of adherent single cells grown in 2D, has been adapted, extended and refined, and protocol variants for the quantification of non-adherent clonogenic growth in 3D using semi-solid matrices developed. This has broadened the range of applications to anchorage—independent, growing, self-renewing cells as well as cells that require embedding in extracellular matrices.

Irrespective of the assay format, for certain cell model systems, it can be challenging to determine the ideal—or at least near-ideal—culture conditions for clonogenic growth in vitro. In particular, the clonogenic potential depends on the culture medium composition and the growth requirements of isolated cells at low density, which may be fundamentally different from the growth requirements of the same cells at higher density[16]. Moreover, the phenomenon of cellular cooperation (i.e., a cell's ability to stimulate clonogenic survival via paracrine and/or autocrine mechanisms (Box 1)) needs to be considered not only in the experimental setup but also in the mathematical analysis of clonogenic survival experiments[17].

In this Protocol Extension, we provide details of how to carry out clonogenic assays, updating the previous *Nature Protocols* article on this topic[1]. We have used the protocol we describe here to examine clonogenic survival of cancer and nonmalignant cells in response to radio- and chemotherapy in single- and combined-modality settings[17–19].

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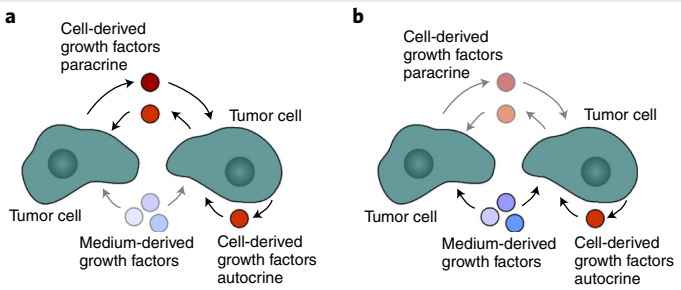
Box 1 | Cellular cooperation

The term 'cellular cooperation' describes the paracrine and/or autocrine stimulation of clonogenic growth by soluble cell-derived factors[17]. This definition was adapted from Puck and coworkers who described that single cells in low density frequently exhibit growth characteristics that are clearly different from those of the same cells in high density, revealing a 'cooperative action between parts of the population differing in genotype or in physiological state'[20]. Importantly, the extent and the impact of cellular cooperation are not only determined by the respective cell type but also by characteristics of the culture medium, including biochemical composition (e.g., concentrations of nutrients, salts or growth factors) and biophysical properties (i.e., medium viscosity, volume per cell, etc.).

The degree of cellular cooperation in a given cell model system can be estimated from the term describing the relationship between the number of single cells seeded (*S*) and the number of colonies obtained (*C*), specifically from the exponent *b*:

$$C = a \times S^b$$

In the absence of cellular cooperation, the growth requirements of all clonogenic cells are fully covered by the culture medium and any culture substrate, and the contribution of cell-derived factors is of minor importance (right-hand image). Accordingly, the relationship between *S* and *C* is linear as reflected by *b* values of ~1. However, quite frequently the formulations of standard cell culture media are suboptimal for single-cell growth, and clonogenic survival depends on the presence and concentration of cell-derived factors, which in turn depend on the initial cell density seeded (left-hand image). In these scenarios of cellular cooperation, the relationship between *S* and *C* fails to be linear, and a constant plating efficiency (PE), which is independent of the cell density seeded, cannot be determined. Mathematically, this is reflected by *b* values of ≥1.2. Even more than quadratic relationships between *S* and *C* have been observed (*b* > 2), indicating cellular cooperation at extraordinarily high degrees[17].



The impact of medium- and cell-derived growth factors on clonogenic single-cell growth under conditions with a high (**a**) or low (**b**) degree of cellular cooperation. Image reprinted from ref. [17].

Determining the degree of cellular cooperation

As an integral part of the pre-experimental procedure and as soon as optimized assay parameters have been developed, the degree of cellular cooperation in a specific assay format needs to be determined. To this end, single-cell suspensions are plated at different cell densities, resulting in a countable number of colonies for control conditions and the treatments of interest[17,27,28]. Subsequently, the relationship between *S* and *C* is fitted by using power regression (i.e., linear regression on log-transformed values) according to $C = a \times S^b$. This can be readily performed by using standard statistical software, the MS Excel template file provided with this protocol (Supplementary Table 1) or the R-package CFAcoop (https://cran.r-project.org/web/packages/CFAcoop).

Cellular cooperation and its relevance for choosing the appropriate analysis algorithm for the clonogenic assay experiment

Depending on the degree of cellular cooperation and the *b* values obtained in the pre-experimental procedure, the appropriate analysis algorithm and the resulting setup of the clonogenic assay experiment are chosen (Fig. 2a). Generally, we recommend relying on power regression, because this algorithm accounts for the impact of cellular cooperation (even if it is of minor degree) and provides results of improved robustness. It also counterbalances systematic errors emerging from sub-linear relationships between *S* and *C* that may occur in specific settings[17,33–35]. Nevertheless, in cases of a linear or near-linear relationship between *S* and *C* (0.8 < *b* value < 1.2) where the impact of cellular cooperation is of minor importance, PE-based normalization may be used in terms of convenience.

Development of the clonogenic assay protocol

Only a few years before the first clonogenic assay was published, mammalian single-cell growth was considered impossible because the formulations of culture media available at that time were not suitable to sustain cell growth at low density. The development of the clonogenic assay became technically feasible when Sanford and colleagues recognized that single-cell proliferation could be achieved by using conditioned media from high-density cultures to grow colonies of single-cell origin in tiny capillaries where diffusion of cell-derived factors was restricted[16].

This inspired Puck and Marcus to develop a procedure in which low-density cell cultures were supplied with sufficient amounts of cell-derived growth factors to allow clonogenic growth[4]. Because they found that some of these factors were unstable in culture, they used a layer of radiation-sterilized feeder cells to condition the medium for single-cell survival in the same dish. The growing colonies

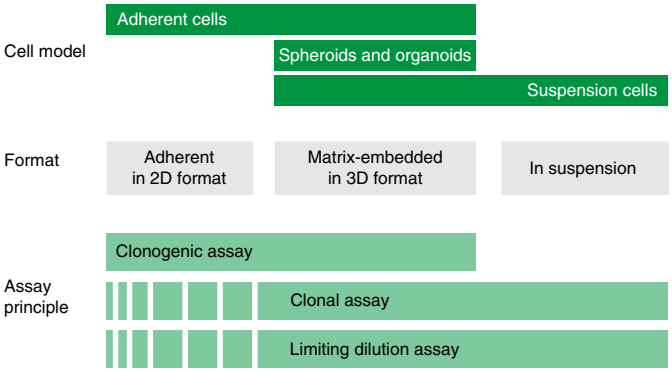


Fig. 1 | Overview on the different assay formats and assay principles to determine clonogenic growth.

eventually became self sustaining, and clonogenic growth with nearly 100% plating efficiency (PE), defined as the number of clonogenic cells divided by the number of single cells initially seeded, was achieved. Puck and coworkers concluded that single cells needed to be supplied with sufficient amounts of cell-derived factors from feeder layers and described the 'cooperative action' of cells under low-density culture conditions to be crucial for clonogenic growth[20]. In successive experiments, they refined the single-cell seeding procedure, rendering feeder layers dispensable in certain contexts. Nevertheless, they clearly emphasized the need to optimize single-cell growth conditions for any cell type of interest[2,3,21].

Since then, 2D clonogenic assays with adherent cells have been frequently used, and this technique enabled fundamental oncological and radiobiological discoveries[11,22–24]. The need for a method to determine clonogenicity was obviously not restricted to cells growing adherently in 2D in vitro, and the protocol was readily adopted by pioneering stem cell researchers who used in vivo assays to determine the frequency of colony-forming hematopoietic cells in the spleens of recipient mice[25,26]. Furthermore, the necessity to measure anchorage-independent clonogenic growth led to the development of in vitro clonogenic assay formats for 3D embedded cells in the 1970s[27–29]. In this setting, nutrient and growth factor supplementation was provided by a bottom layer of semi-solid culture medium (i.e., culture medium supplemented with gellants, such as agarose, agar agar, methylcellulose or, more recently, extracellular matrix preparations), and single cells were allowed to grow to 3D spheroids or organoids in a second layer of semi-solid medium, which both limits diffusion of cell-derived factors and restricts dispersion of embedded cells from growing cell clusters. Importantly, this modification of the initial protocol by Puck and Marcus extended not only the spectrum of cell model systems that could be applied to clonogenic assay formats in vitro (Fig. 1), but also the range of research questions that could be addressed. Whereas adherent 2D colony growth is an important feature for assessing the cytotoxic effects of diverse treatments in established mammalian cell lines and other cell model systems that can be grown in adherent monolayers, matrix-embedded colony growth has often been used to measure the frequency of anchorage-independent, growing stem(-like) cells capable of self-renewal and/or differentiation. Accordingly, the methodology to detect colony-forming cells in ex vivo preparations of various tissues in vitro essentially enabled the characterization of rare stem(-like) cells within heterogeneous populations of different origins[5,6,15,30–32]. In addition to the clonogenic assay, technically related assay principles that we herein refer to as the clonal assay and the limiting dilution assay are available (Fig. 2). These are commonly performed with 3D embedded single-cell preparations or in suspension. Similar to the clonogenic assay, they were developed to detect and quantify clonally growing cells with self-renewing capacity in vitro, but their conceptual assay designs and technical readouts are different (see Comparison between cell viability, clonal and limiting dilution assays).

The clonogenic assay has traditionally been analyzed by PE-based normalization; that is, the fraction of clonogenic cells surviving upon a given cytotoxic treatment is divided by the fraction of clonogenic cells under control conditions. It is important to stress that the robustness of this kind of normalization relies on the constancy of the PE as indicated by a linear relationship between the number of cells seeded (S) and the number of colonies obtained (C)[17]. However, various studies have revealed that the PE of a relevant proportion of cell culture models is not as constant as it has been considered to be[17,33–36]. It can be strongly influenced by different assay parameters. As such, the total assay volume and the cell density seeded are of critical importance[17]. The underlying reasons (i.e., the

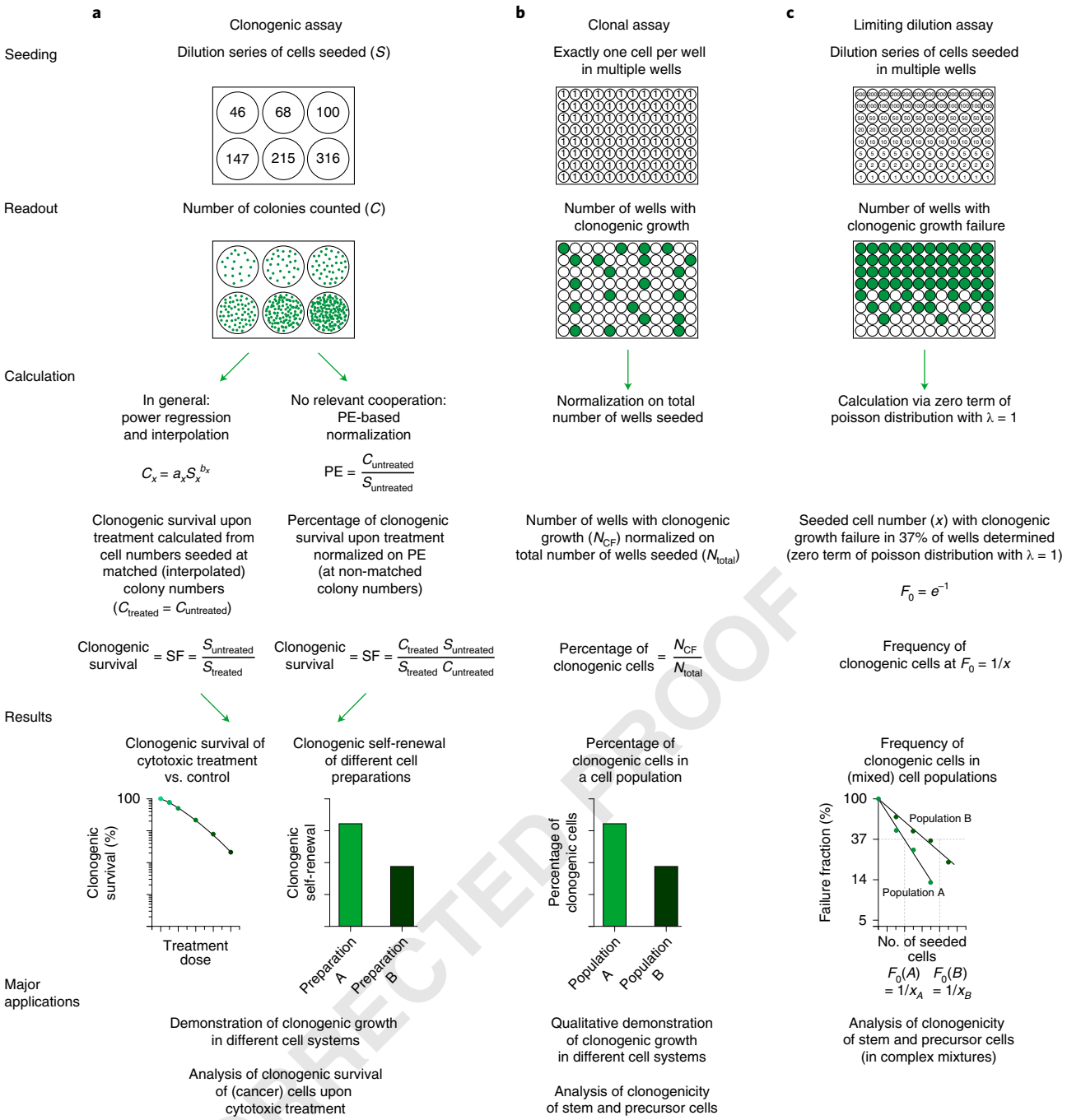


Fig. 2 | Comparison of different assay principles to assess clonal cell growth. a, Clonogenic assays are typically performed in culture dishes with surface areas of clearly more than 1 cm^2 (e.g., six-well plates). Depending on the cell model system and the treatment(s) of interest, the number of single cells seeded in the same well (*S*) can vary from <10 to several thousands. After an incubation period sufficiently long for clonogenic growth, the colony counts (*C*) in each well are determined. Because multiple cells are seeded into each well, cellular cooperation is frequently observed. Two different mathematical approaches for the calculation of SFs relative to controls exist as discussed in detail in the main text: power regression–based analysis and PE-based normalization. Depending on the scientific question of interest, the results are displayed as clonogenic survival upon cytotoxic treatment (at different doses), or a comparison of clonogenic self-renewing capacity of different cell preparations is shown. **b** and **c**, Similar to clonogenic assays, clonal assays (**b**) and limiting dilution assays (**c**) are used to determine the frequency of clonal single-cell growth. In contrast to the clonogenic assay, these are usually performed in smaller multi-well formats, such as 96-well plates. In the clonal assay (**b**), each well is equipped with exactly one single cell, thus excluding the occurrence of cellular cooperation. To determine the percentage of clonogenic cells in a population, the number of wells with clonal growth (N_{CF}) is normalized to the total number of cells seeded (N_{total}). The primary readout of limiting dilution assays (**c**) differs from the other two assay principles. Here, multiple cell densities per treatment condition are plated to determine the fraction of wells with colony-formation failure. With the use of Poisson statistics, the frequency of clonal cells in the cell population of interest can be inferred from the failure fraction (*F*) according to the zero term of the Poisson distribution with the expected value $\lambda = 1$.

characteristics of the chosen culture medium and the phenomenon of cellular cooperation (Box 1)) were well known to the pioneers in the field of low-density cell culture and clonogenic survival but moved out of scientific awareness afterwards[2,3,20,21]. In consequence, the nonconstancy of the PE and its impact on the robustness of clonogenic survival analyses have been largely underestimated for a long time. In clonogenic assays with cooperatively growing cell model systems, PE-based normalization generates large assay-intrinsic errors, and alternative analysis algorithms that can account for the impact of cellular cooperation need to be used[17]. The present protocol provides a mathematical approach involving power regression and interpolation to address this issue. Although the prerequisite for this analysis workflow does not exceed simple linear regression (on log-transformed values), it would have been harder to implement in the 1950s when survival data were commonly fitted by hand. Nevertheless, it is inspired by Puck's recommendations. In principle, it calculates how many times more cells need to be seeded for a given condition to obtain identical numbers of colonies to those seen under control conditions and thus corrects for varying degrees of cellular cooperation in different dishes of the same experiment. This is fundamentally different from the PE-based workflow whose use should be restricted to cell model systems that do not exhibit cooperative growth behavior (Fig. 2a).

Applications

The clonogenic assay can be undertaken on a wide spectrum of mammalian cells, including continuously growing established cancer cell lines, low-passage-number patient-derived tumor explant cultures, purified primary cells and mixtures of cells from ex vivo tissue preparations. However, two crucial conditions must be met. First, at least a fraction of cells within the population of interest needs to be capable of undergoing a minimum of six cell divisions in vitro, giving rise to colonies of ≥50 cells. Second, (near-perfect) single-cell suspensions must be able to be generated from the cell population of interest. The latter can usually be achieved by the use of cell strainers and enzymatic or mechanical dissociation of the cells. In contrast, the capacity of clonal growth may greatly depend on the cell type as well as the in vitro culture conditions, and their optimization can be the most challenging part of a clonogenic assay. Whereas satisfactory levels of clonogenic growth can be commonly achieved for established cancer cell lines by using standard culture media as recommended by the suppliers, more elaborate cell model systems may have complex requirements. For instance, single cell–derived colonies of epidermal keratinocytes in vitro were obtained in adherent 2D coculture with feeder cells at optimized density in basal medium supplemented with calf serum and hydrocortisone[5]. In contrast, clonogenic in vitro growth of neural stem cells isolated from adult brain tissue was first described by using nonadhesive culture dishes and serum-free medium supplemented with epidermal growth factor[6]. These examples show that the applicability of the clonogenic assay largely depends on whether suitable culture conditions for the colony-forming cells in the population of interest can be identified. Particularly for cell mixtures of ex vivo tissue preparations, this may be technically demanding but eventually possible. With emerging research interest in a given field, commercially available culture media are being increasingly developed to facilitate this, even for cell types with complex growth requirements[37].

Comparison between cell viability, clonal and limiting dilution assays

Unlike widely used cell viability assays, which assess the metabolic activity, reductive capacity and/or energized status of cells[38–42], the clonogenic assay detects only the proliferating fraction of cells that is able to form colonies with ≥50 cells. This corresponds to a minimum of six cell divisions and is considered as a surrogate for long-term, 'unlimited' proliferation. Commonly, clonogenic assays are more time consuming and laborious than viability assays, and they can barely be automated. Nevertheless, in various settings, it may not only be advantageous but even essential to quantify only cells with long-term, 'unlimited' replicative potential instead of cell viability alone—for instance to assess an in vitro relapse upon treatment with anti-cancer therapy or with the aim of identifying stem (-like) cells in cell suspensions of ex vivo tissue preparations.

The primary readout of clonogenic assays is typically obtained by counting the numbers of colonies that develop in different culture dishes (Fig. 2a). This raw dataset is subsequently used to calculate the fraction of clonogenic cells as discussed below. Other assays that determine the cellular self-renewing capacity with similar yet distinct conceptual setups and analysis workflows are clonal and limiting dilution assays (Fig. 2b,c). For these assays, the primary readout of clonality is binary because they distinguish only between samples with or without clonal growth, often followed by

morphological, biochemical and/or surface marker characterization of the colony-forming cells. Accordingly, these assays are typically performed in smaller multi-well formats, such as 96-well plates, with multiple wells per treatment condition. In the assay principle that is commonly referred to as a 'clonal assay', a single cell per well is cultivated (e.g., by using micro-manipulation techniques), and the number of wells exhibiting clonal growth is determined under a (stereo-)microscope[43–45] (Fig. 2b). Although this approach certainly measures bona fide clonality and naturally excludes cellular cooperation between different cells of the donor population, it is very laborious and not practical if the frequency of colony-forming cells is in the lower or sub-percentage range. In these scenarios, the 'limiting dilution assay' is an alternative. It is particularly suitable for inferring frequencies of colony-forming cells in heterogeneous cell mixtures (e.g., in ex vivo tissue preparations). It was developed initially for the study of hematopoiesis and interrogates the occurrence of clonal growth in serial dilutions of individual wells with different numbers of single cells seeded[46–49] (Fig. 2c). The limiting dilution assay may be helpful if a classical clonogenic assay with multiple colonies grown in the same well is not feasible, not desired or excluded due to other reasons. Mathematically, the analysis of limiting dilution assays relies on determining the failure fraction of wells without clonogenic growth according to the Poisson distribution with an expected value $\lambda = 1$. An easy-to-use online tool is readily available to facilitate the analysis[50] (http://bioinf.wehi.edu.au/software/elda/).

Experimental design

The basic stages of the clonogenic assay are summarized in Fig. 3. Most importantly, two different plating formats—adherent 2D growth and embedded 3D growth—are available. Moreover, if the impact of cytotoxic treatments on clonogenic survival is tested as described below, it is additionally necessary to choose between one of two plating options. In option A (pretreatment plating), a single-cell suspension of a given cell type is generated from one donor culture, seeded into six-well plates (or culture dishes), allowed to adhere and finally subjected to the treatment(s) of interest. In option B (posttreatment plating), several donor culture flasks are subjected to the treatment(s) of interest, and plating is performed either immediately afterwards or after a given time period, respectively. For both options A and B, plates are filled with identical volumes of culture medium per well and are subsequently incubated in a humidified CO_2 incubator at physiological temperature for sufficient time to allow surviving cells to form colonies of ≥50 cells (a minimum of six population doublings). Finally, colonies are fixed, stained and counted before mathematical analysis is performed.

2D and 3D plating formats of clonogenic assays

Adherent 2D growth is the most simple and most rapid technique that is commonly used for adherently growing cell model systems, whereas 3D embedding of single cells in a semi-solid matrix may be the option of choice if adherent clonogenic cell growth is either not possible or not desired. In Reagent setup, we describe the use of agarose as gellant for embedded 3D clonogenic assays allowing and/or requiring anchorage-independent growth, a feature of many stem(-like) cells. Moreover, agar agar[30,51,52] and methylcellulose[31,32,53,54] have been used for this purpose. Similarly, preparations of extracellular matrix allow embedded 3D colony growth[55]. This 3D assay format may be useful for anchorage-dependent cells that are unable to grow in agar(-ose) and/or methylcellulose.

Numerous variations of the 3D embedded growth protocol exist. Usually, the single-cell suspension of interest is plated onto a semi-solid bottom layer that may improve nutrient supplementation. Moreover, growth-promoting or cytotoxic agents can be added on top of (or into) the semi-solid matrix layers after (or during) plating (see Treatment options and Facilitation of colony growth).

Further information on the advantages and limitations of the two assay formats is provided in Table 1.

Pretreatment plating (option A) versus posttreatment plating (option B)

Regarding the two common alternative plating options, it should be emphasized that both pre- and posttreatment plating (Fig. 3) have distinct advantages in certain settings and, conversely, may be less useful in others. Pretreatment plating usually requires less time than posttreatment plating if several different conditions are tested (e.g., four to six different doses of ionizing radiation). Only one donor culture flask and one dilution series thereof are necessary to equip all plates (condition(s) of interest and controls) with the required numbers of seeded cells for one biological replicate. Yet, treatment

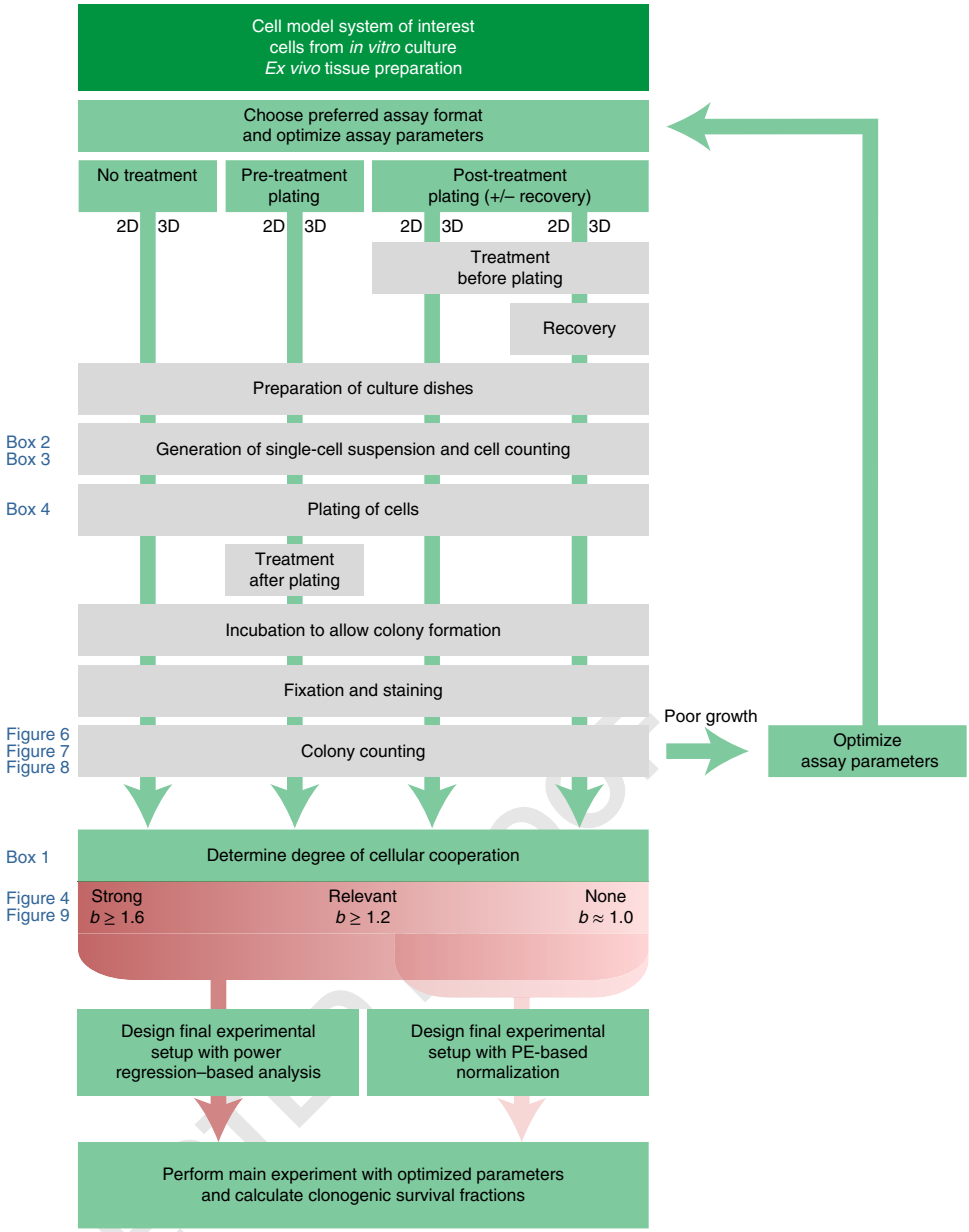


Fig. 3 | Schematic overview of the different clonogenic assay procedures.

may be time consuming, for instance, when a large number of plates needs to be subjected to radiation.

Conversely, posttreatment plating may be useful when many plates with one or very few identical treatments are needed (e.g., treatment with only one dose of ionizing radiation versus control and subsequent addition of pharmacological inhibitors). In this case, it is clearly less laborious to treat large numbers of cells in separate culture flasks before preparing single-cell suspensions and the derived dilution series to equip all plates (condition(s) of interest and control) of one experiment. However, this plating protocol has an additional source of error that needs to be considered. Here, the single-cell suspensions for each condition and for the controls as well as the dilution series thereof are prepared independently from different (pretreated) donor cultures. This can give rise to relevant unwanted variability in cell count, cell aggregation, cell adhesion, etc. Because of the resulting errors, posttreatment plating may be inappropriate to detect relatively small survival differences between treatments. For instance, the error of the counting procedure with a hemocytometer has been reported to exceed 20% if only one count for each cell suspension is performed[56].

Table 1 | Advantages and disadvantages of 2D and 3D clonogenic assay formats

	Adherent 2D growth	Embedded 3D growth
Advantages	Easy and rapid seeding	More physiological growth conditions
	Less expensive than 3D embedded growth	Biophysical limitation of cellular cooperation
	Rapid colony counting	Increased colony density because of reduced cell migration
Disadvantages	Clonogenic potential may be abrogated by adherent growth (loss of stemness)	Clonogenic potential may be abrogated by embedding
	Cellular cooperation occurs frequently	More time and consumables required
	Cell migration may result in dispersed colony growth	Potential cell stress because of overheating (hot liquid agarose solution) and/or cooling (agarose gelling)
	Limited in vivo relevance (2D growth)	Complex counting procedure or surrogate readout (colony diameter instead of cellularity)

Treatment options

Various treatments, including ionizing radiation, cytotoxic drugs, molecularly targeted agents, hyperthermia, nanoparticles, genetic manipulation, hydrostatic pressure, extracellular vesicles, killing by cytotoxic immune cells and combinations thereof, have been tested in clonogenic assays[7–14,53,57–59].

Depending on the cell model system and the severity of the treatment(s) of interest, the number of *S* per dish may range from $<10^1$ to $>10^5$ cells per well. In the case of widely-used established cell lines, the fraction of cells with clonogenic self-renewing capacity under suitable conditions without cytotoxic treatment is commonly in the double-digit percent range. Nevertheless, clonal growth of more complex cell model systems and particularly cell suspensions from ex vivo tissue preparations may be clearly below 1%, particularly after severe cytotoxic treatment, implying that the assay may not be suitable for some applications.

With regard to combined-modality treatments, the treatment sequence may be of importance. For instance, when combining a chemotherapeutic drug and ionizing radiation, the drug can be added before or after irradiation. It can be removed after a certain time or left with the cells during the full assay incubation period. In this scenario, the half-life of the drug in the culture medium as well as its concentration in biologically relevant settings should be considered. If clonogenic survival is exceedingly low, dose reduction and shortening of the drug incubation period may be helpful. In general, seeding of single cells for adherent 2D growth in the presence of a potentially damaging drug should be avoided because this may compromise the adhesion process. Moreover, additional monotherapy control plates may be necessary for reasonable comparisons between treatments and analyses of synergism. In 3D assay formats, soluble agents of interest can be integrated into at least one of the semi-solid phases during plating. Alternatively, aqueous drug solutions can be added onto the upper gel layer (once or repeatedly).

Technical and mathematical assay optimization

We strongly recommend that preliminary assays be undertaken to optimize the key parameters before setting up all the experimental conditions required for the main experiment. This optimization includes (i) finding an appropriate method for the generation of (near-perfect) single-cell suspensions (Box 2 and 3), (ii) defining suitable parameters that allow clonogenic growth under all conditions of interest (see Facilitation of colony growth, Reagent setup and Box 4) and (iii) assessing the degree of cellular cooperation (Box 1).

As soon as the pre-experimental procedure has been successfully completed, the optimized assay parameters can be used for the main experiment. Actual experiments are typically performed by using three or more independent biological replicates. At this step, the appropriate mathematical analysis algorithm for the calculation of survival fractions (SFs) needs to be chosen (Fig. 2a and Box 1). If the impact of cellular cooperation is negligible, SFs in response to the treatments of interest can be calculated by normalization to the PE of the untreated controls:

$$PE = C_{untreated}/S_{untreated}$$

$$SF_{treated} = \frac{C_{treated}/S_{treated}}{\mathrm{PE}}$$

Box 2 | Generating single-cell suspensions from cells of different origin

To generate single-cell suspensions from cells grown in vitro, follow option A below. Use option B to generate single-cell suspensions from ex vivo tissue preparations. We use option A for A-549 and EFM-19 cells.

Clonogenic assays are designed to quantify clonal single-cell growth. Therefore, it is crucial that the cells are plated in single-cell suspensions without any aggregates. Plating of cell clusters instead of single cells undermines the ability to measure truly clonogenic growth and results in overestimation of SFs at low to intermediate treatment doses[73,74]. Depending on the origin of the cells to be tested, very different protocols may be used to obtain (near-perfect) single-cell suspensions of which two common options are briefly described in this box. Suspension cell lines and suspensions of ex vivo preparations (e.g., hematopoietic cells or cells from effusions) often contain few or no cell aggregates. Nevertheless, mechanical and/or enzymatic disruption of cell clusters may also be achieved by using the techniques described below.

(A) **Generation of single-cell suspensions from adherent cells grown in culture** ● **Timing 10–45 min**

Additional materials required

- Cell detachment reagent, such as 0.05% (wt/vol) trypsin/EDTA (0.5% (wt/vol) stock solution (e.g., from Thermo Fisher Scientific, cat. no. 15400054) can be diluted 1 + 9 in PBS) or TrypLE Express (Thermo Fisher Scientific, cat. no. 12604039). We use 0.05% (wt/vol) trypsin/EDTA for A-549 and EFM-19 cells.
- (Optional) Cell strainers, e.g., 30-μm mesh size (Miltenyi, cat. no. 130-110-915)

Procedure

1 Remove culture medium from the donor culture and wash cells twice with PBS.
2 Remove PBS and add the detachment agent (e.g., 1 ml of 0.05% (wt/vol) trypsin solution per 60-cm^2 flask surface for A-549 and EFM-19 cells) without damaging the cells. Ensure that the cell detachment reagent covers the entire bottom of the flask.
3 Incubate at 37 °C. Repeatedly check whether cells round up and detach by using a microscope. Depending on the cell type, washing procedure and cell density, cell detachment takes 3–30 min and may be accelerated by gently tapping the side of the culture flask. We find it usually takes 8–15 min for A-549 and EFM-19 cells.
4 Inactivate cell detachment reagent by adding a sufficient amount of FCS containing culture medium or a trypsin inhibitor solution. Transfer the cell suspension to a sterile 50-ml centrifuge tube.
5 Generate viable single-cell suspensions by using a method optimized for your cells. For example, we triturate the A-549 or EFM-19 cell suspension several times with a serological pipette to dissociate cell aggregates. Avoid formation of air bubbles.
6 Using a microscope, check if a (near-perfect) single-cell suspension was obtained. If necessary, proceed with other measures for the generation of a single-cell suspension of the given cell type, such as centrifugation of the cells (300*g* and 5 min are suitable for most cell types) and subsequent washing in 20 ml of PBS. In case of strong cell aggregation, cell strainers with mesh sizes of ≤30 μm may also be helpful. Ensure that the suspension is free from cell aggregates before proceeding. We usually obtain a (near-perfect) single-cell suspension for A-549 cells without other measures than those described in step A(v). For EFM-19 cells, we additionally wash the cells in 20 ml of PBS and triturate several times.
? TROUBLESHOOTING
7 Resuspend the single-cell suspension in culture medium and proceed with cell counting.

(B) **Generation of single-cell suspensions from ex vivo tissue preparations** ● **Timing several hours, depending on the digestion protocol**

Additional materials required

- (Optional) Sterile scalpels (e.g., neoLab, cat. no. 1-1409), tissue homogenizers (e.g., Miltenyi, cat. no. 130-095-937) or cell strainers with 30–100-μm mesh size (e.g., Miltenyi, cat. nos. 130-110-915 and 130-098-463)
- Tissue-specific digestion kit or customized combination of tissue digestion reagents, such as collagenase, hyaluronidase and DNAse preparations (an overview can be found at https://www.sigmaaldrich.com/DE/de/products/protein-biology/proteins-and-enzymes)

Procedure

1 Aseptically transfer the ex vivo preparation of tissue to a fresh culture dish or tube containing PBS or suitable culture medium to prevent dehydration.
2 Generate a viable single-cell suspension from the primary tissue preparation. Single cells can be liberated by a combination of mechanical measures (cutting, tissue homogenization, cell straining and repeated pipetting) and enzymatic digestion by using customized enzyme preparations or commercially available tissue type–specific digestion kits. Further enrichment of the cell population(s) of interest can be achieved by cell surface marker–based purification by using magnetic-activated cell sorting or FACS. Ensure that the suspension is free from cell aggregates before proceeding.
3 Using a microscope, check if a (near-perfect) single-cell suspension was obtained. If necessary, proceed with other measures for the generation of a single-cell suspension of the given cell type, such as centrifugation of the cells (300*g* and 5 min are suitable for most cell types) and subsequent washing in 20 ml of PBS. In case of strong cell aggregation, combinations of cell strainers with decreasing mesh sizes may also be helpful. Ensure that the suspension is free from cell aggregates before proceeding.
? TROUBLESHOOTING
4 Transfer the single-cell suspension to a sterile 50-ml centrifuge tube.
5 (Optional) Wash the cells in 20 ml of PBS.
6 Resuspend the single-cell suspension in culture medium and proceed with cell counting.

This mathematically simple approach can be advantageous, because only a few (typically one to three) different cell densities per condition need to be plated. If, however, a nonlinear relationship between S and C for any condition of interest is observed, PE-based normalization generates large to enormous systematic errors that are not amenable to statistical error analysis[17]. This is commonly the case for b values ≥1.2. Under these conditions, we recommend power regression-based analysis according to $C = a \times S^b$, which is applicable without restriction because it accounts for the effects of cellular cooperation and thus provides clonogenic survival results of improved robustness. For each treatment condition, this approach requires at least five different S values to be seeded giving rise to C values in the range of ~5–100 colonies. Here, the calculation of SFs does not rely on a fixed PE of the

Box 3 | Cell counting ● Timing 5–10 min

The accurate determination of viable single-cell counts is critical for clonogenic survival experiments. To date, hemocytometers are the cheapest option of manual cell counting for which the Neubauer Improved Chamber is very commonly used. The 3 × 3 mm^2 laser-etched gridded area of this hemocytometer consists of nine squares of 1-mm^2 size (and with properly mounted coverslip, 100-nl volume) each. Nucleated mammalian cells are typically counted in the four corner squares (Fig. 5e,f): a coverslip is mounted onto the chamber, the cell suspension is loaded by capillary action and the cell count is determined as described below. Exclusion of dead cells is achieved by mixing the single-cell suspension with, for instance, trypan blue, a vital stain that is exclusively taken up by dying and dead cells.
Automated alternatives to hemocytometer-based manual cell counting are available. Although more expensive, these devices facilitate rapid and reliable cell counting. They use different detection principles, such as resistive pulse sensing, photo-microscopy with integrated digital analysis and flow cytometry–based optical analysis with laser detection[72]. In specific settings, the latter technique may be particularly relevant for the analysis of colony-forming cells because it allows the combination of cell counting with FACS of relevant subpopulations in a cell suspension[31,53].

Manual cell counting with a Neubauer Improved Chamber
Additional materials required
- Small aliquot of the single-cell suspension as generated according to Box 2
- 0.4% (wt/vol) trypan blue solution (e.g., Sigma-Aldrich, cat. no. T8154)
  ! CAUTION Trypan blue is toxic. Handle with care and always wear protective gloves.
- Neubauer Improved Chamber (e.g., BRAND, cat. no. 717805)

Procedure
1. Prepare two independent 1:1 dilutions of the single-cell suspension of interest with 0.4% (wt/vol) trypan blue and mix gently (e.g., 40 µl of suspension + 40 µl of trypan blue in a fresh microcentrifuge tube). Incubate for 1–2 min at room temperature.
2. Prepare the Neubauer Improved Chamber by making the coverslip and chamber humid and mounting the coverslip properly onto the chamber. Newton's rings should be visible.
3. Load 10 µl of the diluted single-cell suspension to each side of the chamber by using a 2–20-µl micropipette and check if the coverslip is still in place. Avoid air bubble formation.
   ? TROUBLESHOOTING
4. Count the viable cells within the four corner squares at 40-fold magnification. Exclude dark blue (i.e., trypan blue–positive) cells and debris. Count only cells touching two of the four outer grid lines of each 1-mm^2 square and note the total cell count of both chambers.
   ? TROUBLESHOOTING
5. Clean the Neubauer Improved Chamber in 70% (vol/vol) ethanol and let dry. Repeat steps 2–4 to obtain two more counting results.
6. Determine the average cell counts of all sixteen 1-mm^2 squares from four independent countings.
7. Calculate the concentration of viable single cells with the following formula. Consider the dilution factor of 2 for the 1 + 1 dilution in trypan blue. Note that the total volume in one 1-mm^2 square is 100 nl.

$$c = \frac{Mean\ viable\ cell\ count\ of\ all\ 1\ mm^2\ squares}{100\ nl} \times dilution\ factor$$

untreated controls. Instead, clonogenic survival is obtained via power regression and interpolation of matched colony numbers under untreated and treated conditions (Fig. 4).

To eliminate the error that originates from violating the linearity assumption for *S*-*C* variable pairs, the SFs resulting from the condition(s) of interest are calculated by determining $S_{untreated}/S_{treated}$ ratios of matched colony numbers ($C_{untreated} = C_{treated}$). Power regression according to $C = a \times S^b$ is used to model the number of *C* in dependence of the number of *S* and to estimate the corresponding parameters *a* and *b* (Fig. 4a). With these parameters, *S* values ($S_{untreated}$ and $S_{treated}$) are interpolated, and SFs at matched colony numbers are calculated via:

$$SF_{treated} = \frac{S_{untreated}}{S_{treated}} = exp\left(\frac{log\left(\frac{C}{a_{untreated}}\right)}{b_{untreated}} - \frac{log\left(\frac{C}{a_{treated}}\right)}{b_{treated}}\right)$$

We suggest performing the calculation for $C = 20$ (Fig. 4b), reflecting a number of colonies that can typically be observed under various treatment conditions in clonogenic assays. For a detailed derivation of the formula, see Brix et al.[17].

An illustration of the entire assay optimization procedure and an actual experiment with an established human breast cancer cell line is depicted in Fig. 5.

Facilitation of colony growth

For various cell model systems, particularly primary cells, standard culture media may be suboptimal with regard to single-cell growth. This is usually reflected by poor colony growth even in complex media, which is often accompanied by very high degrees of cellular cooperation (Box 1). In specific settings, it might therefore be advisable and/or necessary to modify single-cell culture conditions to obtain sufficient colony growth under all relevant treatment conditions of interest. This can be

Box 4 | Single-cell plating

Use the single-cell plating technique compatible with the selected assay format, ensuring that cells remain in a single-cell suspension (prepared according to Box 2). Proceed with option A below for adherent 2D growth. Follow option B for embedded 3D growth.

(A) **Plating of cells for adherent 2D growth in liquid medium** ● **Timing 20 min + cell adhesion time**

Additional materials required

- Culture dishes with liquid medium, as prepared according to Reagent setup
- 50-ml centrifuge tubes with single-cell suspensions of appropriate dilutions for plating as generated according to Box 2 and 3

Procedure

1 Remove the prefilled six-well plates from the incubator.

2 Using a micropipette and the single-cell suspensions of appropriate concentrations, add the indicated number of cells into the respective wells containing 2 ml of medium. Unequal final volumes at this stage are acceptable if the culture medium is refreshed before starting the main incubation step.

3 Distribute the cells evenly by moving the six-well plates several times in the x-y direction. Check homogeneous distribution under the microscope.
▲ **CRITICAL STEP** When cells are not distributed evenly, colony counting will be compromised.

4 Place the six-well plates into a humidified incubator for adhesion (physiological temperature and culture medium–matched CO_2 concentration). Depending on the cell type, this may take 2–20 h. Generally, 3–4 h are required for A-549 cells and 4–6 h for EFM-19 cells. Additional control plates may be helpful to enable regular checking under the microscope whether cells are adherent. Proceed as soon as adhesion of the vast majority (>90%) of cells is completed. Avoid exceedingly long adhesion times if subsequent treatment of single cells is desired because these may rapidly restart proliferation after plating. Treatment of cell clusters instead of single cells results in overestimation of SFs at low to intermediate treatment doses [73,74].
? TROUBLESHOOTING

(B) **Plating of cells for embedded 3D growth** ● **Timing 30 min + time for matrix gelling**

Additional materials required

- Liquid 4% (wt/vol) agarose stock solution (or other desired embedding matrix) and culture dishes with a bottom layer of semi-solid medium prepared as described in Reagent setup
- 50-ml centrifuge tubes with single-cell suspensions of appropriate dilutions for plating, generated as described in Box 2 and 3

Procedure

1 Keep agarose solutions and single-cell suspensions at 37 °C. Dilute all single-cell suspensions 9 + 1 with 4% (wt/vol) agarose. Mix the resulting suspensions containing 0.4% (wt/vol) agarose and immediately store at 37 °C.
▲ **CRITICAL STEP** A heating device is required to keep agarose solutions, culture medium and the single-cell suspensions at 37 °C. Higher or lower temperatures will damage the cells or cause premature solidification of agarose, respectively.

2 Add 1 ml of suitable single-cell suspension to the solid bottom layer of each well of the cooled prepared culture dishes. Avoid air bubble formation. Work quickly to avoid premature agarose gelling.
? TROUBLESHOOTING

3 Distribute the suspension evenly by moving the six-well plates several times in the x-y direction.

4 Allow gelling of the 0.4% (wt/vol) agarose layer containing the embedded single cells on a balanced, flat surface for 30–45 min at room temperature.
? TROUBLESHOOTING

5 Add 100–200 µl of liquid culture medium (optionally containing cytotoxic agent(s) of interest) to each well to prevent dehydration of the gels. This step may be repeated after several days if long assay incubation times are required.

6 Place the six-well plates into a humidified incubator for equilibration (physiological temperature and medium-matched CO_2 concentration). Avoid exceedingly long incubation times if subsequent treatment of single cells is desired because proliferation may restart rapidly after plating. Treatment of cell clusters instead of single cells results in overestimation of SFs at low to intermediate treatment doses[73,74].

achieved with one of the following options or combinations thereof. Note that these additions to the procedure may be rather time consuming, and optimal adaptations are often based on mostly unknown individual requirements of the cell model system of interest.

Optimization of culture medium. The formulations of standard culture media differ considerably with regard to the composition of ingredients and their concentrations[60]. Cell growth of a specific cell type may be greatly facilitated by using another medium formulation and/or additives (such as fetal calf serum, bovine pituitary extract, recombinant growth factors, hormones, etc.). Of note, many additives, such as fetal calf serum and growth factor supplements, may exhibit substantial variability across providers, preparations and batches.

Conditioned media. An enrichment of factors supporting or necessary for clonogenic single-cell growth can be obtained by use of cell-free conditioned media from high-density cell cultures of the cell type of interest or an unrelated cell type that is known to secrete growth-promoting factors suitable for the cell type to be analyzed. Conditioned media have been successfully used since the very first mammalian single-cell growth experiments[16,17]. If standardization of the conditioned media is desired, basic parameters, such as cell density after seeding, cell-to-volume ratio, conditioning time

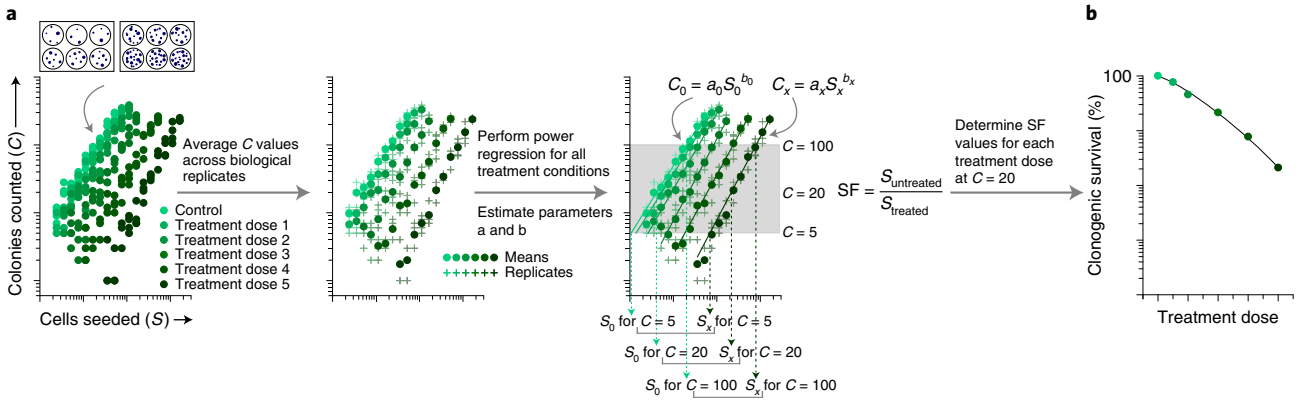


Fig. 4 | Scheme depicting the principle of power regression–based clonogenic survival data analysis. a, For any treatment condition of interest, ≤12 variable pairs of *S* and *C* are acquired. Each single data point in the left panel corresponds to the colony count obtained from one well in a six-well dish of one biological replicate. For controls (S_0-C_0 pairs of values) and each treatment of interest (S_x-C_x pairs of values), *C* values are averaged across all biological replicates, power regression (i.e., linear regression on log-transformed values) according to $C = a \times S^b$ is performed and the parameters *a* and *b* are estimated. Note that any power function appears linear when plotted on a double-logarithmic scale. The SFs for any treatment are calculated by the $S_{untreated}/S_{treated}$ ratios at matched *C* values. We suggest using $C = 20$, because this reflects a colony number that can be typically observed under various treatment conditions. **b**, Clonogenic survival in dependence of the treatment dose as calculated according to *a* for $C = 20$ plotted with semi-logarithmic scaling, standard deviation approximated via error propagation of regression parameters and superimposed linear-quadratic fitting. This scheme was constructed on the basis of a hypothetical dataset.

and storage, should be harmonized. An example of a procedure to generate conditioned media by using the cell line A-549 is given in Reagent setup.

Feeder cells. Similar to conditioned media, the use of feeder cells is intended to supply single cells in a clonogenic assay experiment with sufficient amounts of growth-promoting factors needed for proliferation. Feeder cells can additionally provide cell-to-cell contacts (depending on whether physical barriers between the feeder cells and the cells of interest are used or not). This measure was used in the first clonogenic experiments by Puck and colleagues, and many slightly adapted protocols for these co-culture systems have been developed. Unintentional scoring of feeder cell–derived colonies needs to be excluded. This can be achieved by using feeder cells that are per se unable to form colonies (e.g., due to mitotical inactivation) and/or by avoiding misidentification of colonies due to morphological differences between the two cell types[3,4,54].

Embedding of cells in semi-solid matrices. Whereas assay optimization according to any of the three aforementioned measures results in improved 'external' growth factor supply, it is also possible to limit the extent of diffusion by increasing the viscosity of the culture medium. This enhances the pericellular halo of growth factors produced by the cell type of interest[3]. Although not systematically analyzed yet, this technique is likely to reduce the impact of cellular cooperation. Loss of cell-derived growth factors can be prevented by embedding and/or overlaying single cells by using semi-solid matrices of which agar agar, agarose, methylcellulose and extracellular matrix preparations have been used most frequently. As discussed above, this standard technique has been extensively used for 3D clonogenic assays but was already recommended by Puck and colleagues in very early 2D clonogenic experiments[3,27–29].

Limitations of clonogenic assays

Optimization of assay parameters and colony counting

The pre-experimental procedure of this protocol is intended to develop a clonogenic assay design and optimized culture conditions that allow ideal or at least acceptable clonal growth of single cells. It should, however, be kept in mind that clonogenic growth of a given cell model system can be obtained by using differing conditions, including liquid or semi-solid culture media with varying nutrient and growth factor composition, additives, viscosity, etc. Consequently, clonogenic survival data should be interpreted with care, and the biochemical and biophysical context in which they were generated should be considered. A comparison of survival analyses obtained across different experimental parameters may be misleading, because this type of variability is not accounted for[61].

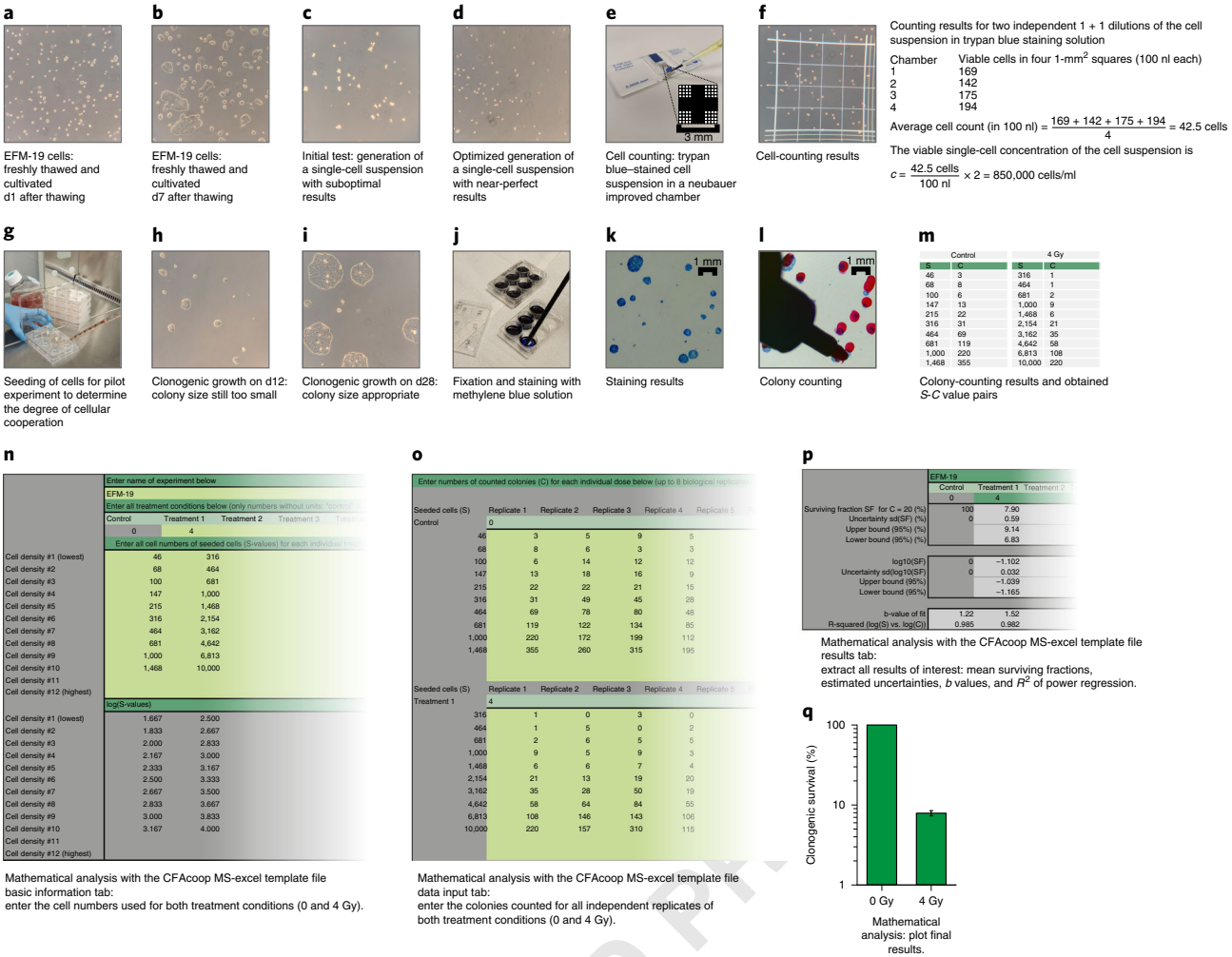


Fig. 5 | An example of the assay optimization procedure for a breast cancer cell line grown in adherent 2D growth. a and **b**, EFM-19 cells (RRID: CVCL_0253) were thawed and cultivated in T175 flasks for 8 d at 37 °C and 5% CO_2 in 1× RPMI 1640 medium with GlutaMAX supplemented with 10% (vol/vol) FCS and 1% (vol/vol) penicillin-streptomycin. The images show the cells on day (d) 1 and d7 after thawing. **c**, An initial test with the cells was made to generate a viable single-cell suspension and to determine whether adherent 2D clonogenic growth can be observed when seeding the cells in their standard culture medium without further additives or embedding. Cells were washed twice in PBS and trypsinized for 8 min at 37 °C by using 3 ml of 0.05% (wt/vol) trypsin. After detachment and gentle agitation of the culture flask, trypsin was inactivated by using 7 ml of culture medium, and the suspension was triturated several times with a serological pipette. Small clusters of cells were observed, indicating that the single-cell nature of the suspension was not suitable for plating. **d**, Cells shown in **c** were transferred to a 50-ml tube, centrifuged (300*g* and 5 min), washed once in 20 ml of PBS and triturated several times. After resuspending the cells in 5 ml of fresh medium, a (near-perfect) single-cell suspension was obtained as shown. **e**, The concentration of viable, trypan blue–negative single cells in the suspension from **d** was determined with a Neubauer Improved Chamber as described in Box 3. **f**, A typical example of a 1-mm^2 square of the loaded chamber is depicted. A concentration of 8.5×10^5 cells/ml was obtained (see the figure for the calculation). 1 ml of this solution (i.e., 8.5×10^5 cells) was diluted in culture medium to a final concentration of 10^5 cells/ml in a fresh 50-ml tube. This suspension was mixed, and serial 1 + 9 dilutions with 10^4 cells/ml and 10^3 cells/ml were generated. **g**, Four six-well culture dishes (2 ml of medium/well) were prepared to examine clonogenic growth as well as the degree of cellular cooperation under control conditions and the treatment of interest (irradiation with 4 Gy and untreated control). Suitable numbers of cells (46–21,500 cells/well) were plated into the dishes and distributed evenly by moving the plates in the x-y direction. Adherence of cells was allowed for 4 h (37 °C, 5% CO_2 and humidified atmosphere), and the great majority of the single cells was adherent afterwards. The medium was refreshed (2 ml/well), and x-ray treatment of 4 Gy plates was performed before starting the incubation period for colony growth. Mock-irradiated cells served as 0 Gy controls. **h**, After 12 d, clonogenic growth on control plates was checked. Several colonies had formed, but these were still very tiny and often below 50 cells, indicating that further incubation of the plates was necessary. **i**, On day 28, EFM-19 colonies of adequate size had developed. **j**, The culture medium was poured off, and EFM-19 colonies were carefully fixed and stained for 1 h at room temperature with 2 ml of 0.8% (wt/vol) methylene blue solution per well. The staining solution was re-collected, and the plates were carefully rinsed in warm tap water and subsequently placed in a drying cabinet (70 °C). **k** and **l**, Colony counting was performed with a stereomicroscope at 8–30× magnification by using a fine-tip lab marker. 10 of the 12 cell densities initially seeded (*S*) resulted in wells with countable numbers of colonies (*C*) for both control and treated plates. **m**, Dataset showing the *S*-*C* value pairs obtained from the counting procedure as shown in **k** and **l** (four independent biological replicates; data taken from ref. [17]). **n**–**p**, Mathematical analysis with the supplied MS Excel template file CFAcoop. **q**, Final graph obtained from the clonogenic assay experiment by using the raw data shown in **m**–**o**.

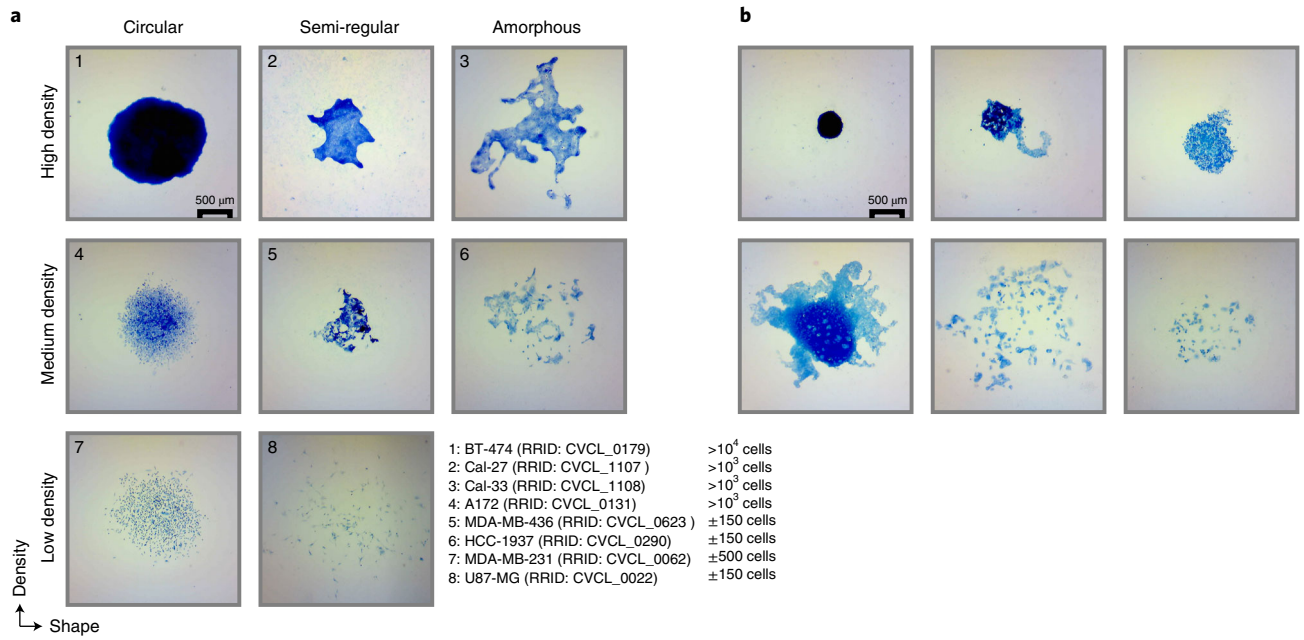


Fig. 6 | Colony morphology displays substantial inter- and intra-cell line heterogeneity. a, Typical colony morphologies of eight different cell lines of breast cancer (BT-474, RRID: CVCL_0179; MDA-MB-436, RRID: CVCL_0623; HCC-1937, RRID: CVCL_0290; MDA-MB-231, RRID: CVCL_0062), head and neck squamous cell carcinoma (Cal-27, RRID: CVCL_1107; Cal-33, RRID: CVCL_1108) and glioblastoma (A172, RRID: CVCL_0131; U87-MG, RRID: CVCL_0022) under untreated conditions are depicted (20× magnification; images were collected from colony-formation assays prepared for ref. [17]). Colonies displayed a high level of inter-cell line heterogeneity with regard to the overall shape of the colony (circular, semi-regular or amorphous) and the density of the colony-forming cells within a colony (high, medium or low). Despite the similar size of all colonies, the overall number of cells within the different types of colonies differed enormously. **b**, Illustration of intra-cell line heterogeneity of colony morphology and size in the pancreatic cancer cell line L3.6pL (RRID: CVCL_0384) under untreated conditions. Circular colonies with high cell density as well as semi-regular and amorphous colonies of different cell density were observed (20× magnification).

This is particularly important when comparing 2D and 3D clonogenic assay formats, which can lead to conflicting results (Table 1)[55,62].

The reliability of clonogenic survival results is further affected by other parameters that cannot be universally standardized. First, the incubation time chosen to allow colony formation is subjective and can in part depend on the experience and the personal preferences of the researcher. Importantly, if insufficient time is allowed for colony formation, the efficacy of a cytotoxic treatment of interest with regard to the abrogation of clonogenic survival can be overestimated. In this case, small and slowly growing colonies that are typically overrepresented on plates with harsh treatment conditions may be overlooked. Conversely, very large and partially confluent colonies resulting from exceedingly long incubation periods will complicate and/or disturb proper data analysis. We recommend optimizing the incubation period in pilot experiments of the pre-experimental procedure. For all conditions of interest, the colony-formation incubation time should be synchronized and be sufficiently long to allow at least six population doublings (even with the harshest treatment) without giving rise to overgrown control plates.

In addition, data quality may be compromised by errors occurring during colony counting. Colonies can vary substantially in size and shape depending on the cell line and the treatment of interest (Figs. 6–8). Even within one cell line and without any further treatment, colonies can have a very diverse appearance (Fig. 6b). Moreover, it may not always be clear whether a given structure of ≥50 cells represents exactly one colony. Fusions between colonies, cell migration phenomena and overgrown plates may render data acquisition difficult (Fig. 7). Finally, the occurrence of huge, morphologically altered, mostly treatment-induced, senescent and/or other forms of giant cells may confound the (non-)clonogenicity of a cell cluster (Fig. 8). Accordingly, we recommend performing colony scoring with the greatest care and with the help of a stereomicroscope. This is of particular importance when using 3D assay formats where colony growth must be analyzed in different layers of a given well[55].

Even experienced researchers may need to practice the critical colony-counting step with every new cell model of interest. Importantly, we strongly discourage using workflows that solely quantify

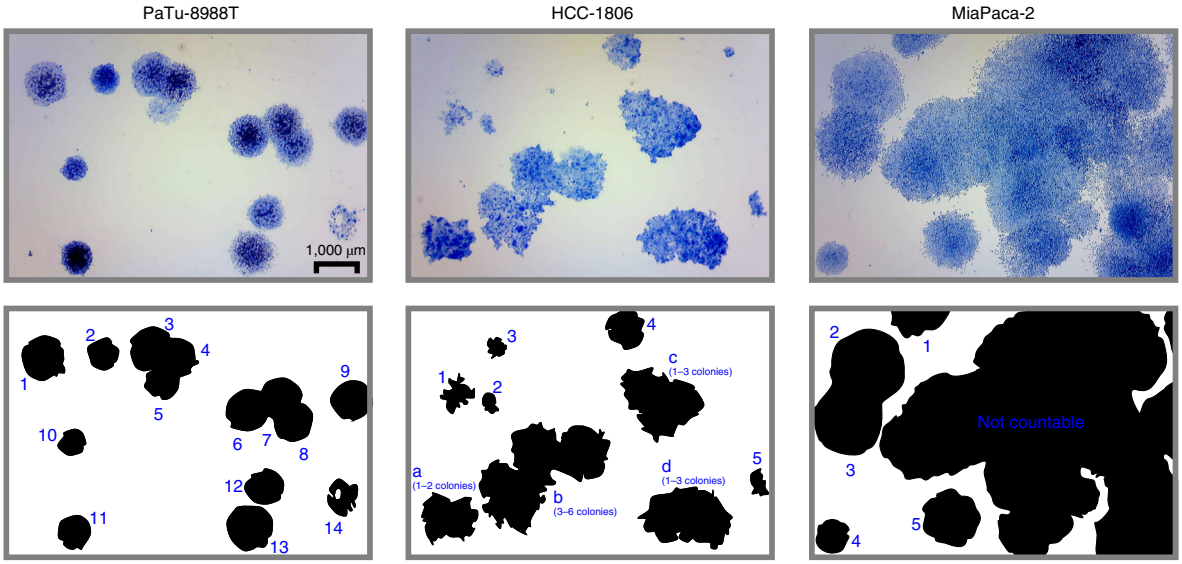


Fig. 7 | High colony density limits the reliability of the colony-counting procedure. Three examples of typical 2D colony growth patterns of untreated cell lines (PaTu-8988T, RRID: CVCL_1847; HCC-1806, RRID: CVCL_1258; and MiaPaca-2, RRID: CVCL_0428) are depicted in the upper panels (10× magnification; images were collected from colony-formation assays prepared for ref. [17]). Corresponding black and white renderings of colony growth are shown below. Whenever possible, cell clusters that were very likely to represent one single colony were numbered in blue. The 14 colonies in the left panel could be scored without difficulty. Quantification of colonies in the middle panel was rather challenging. For the cell clusters a–d, the precise number of colonies was difficult to determine. The colony count for the right panel could not be quantified because it was overgrown.

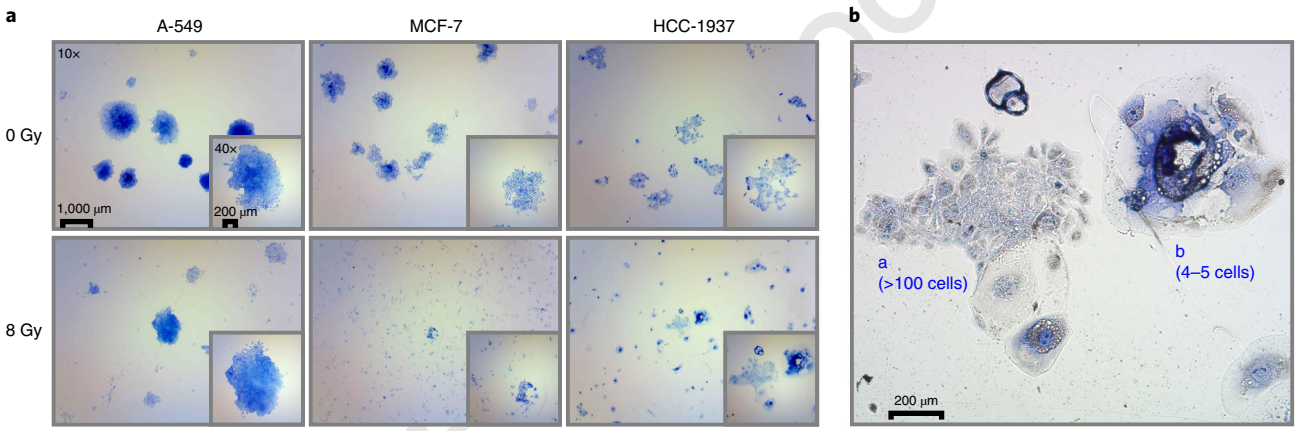


Fig. 8 | Intra-assay heterogeneity adds complexity to accurate colony counting. a, Representative images of colony growth under untreated conditions (upper row, 0 Gy) and upon severe treatment (bottom, 8 Gy) for the lung adenocarcinoma cell line A-549 (RRID: CVCL_0023; left) and the breast cancer cell lines MCF-7 (RRID: CVCL_0031; middle) and HCC-1937 (RRID: CVCL_0290; right) are shown (10× magnification; images were collected from colony-formation assays prepared for ref. [17]). The colony in the center of each image is depicted at higher magnification (40×) in the inlays on the bottom right of each panel. Under untreated conditions, all three cell lines formed colonies of similar size with sufficient staining intensity. Albeit smaller than under control conditions, colonies of irradiated A-549 cells were well detectable. In contrast, the identification of the single MCF-7 colony grown after irradiation (lower panel in the middle) was challenging because its small size and surrounding giant cells. The single colony of the cell line HCC-1937 in the bottom right panel was very difficult to detect at low magnification because of its small size and weak staining intensity. Accurate counting was further complicated by giant single cells with high staining intensity. **b**, Image depicting the colony of HCC-1937 cells shown in **a** at 50× magnification. The small number of giant cells next to the colony is a prominent example of a cell cluster that must not be scored as a colony.

the stained area per well or even solubilize the stained colonies with subsequent colorimetric or fluorometric quantification. Both approaches fail to properly address the challenges arising from inter- and intra-assay differences in cell and/or colony sizes, shapes and staining intensities.

To overcome the—to a certain extent—arbitrary manual counting procedure, automated colony-counting devices and image analysis tools have been developed[55,63–65]. Certainly, these auxiliary means may greatly accelerate the scoring process. Moreover, the decision whether a structure represents a colony or not is based on an objective algorithm. To date, however, it is rather

questionable whether even sophisticated algorithms are able to detect colonies with sufficient specificity and sensitivity under challenging conditions that might occur in clonogenic survival experiments (Figs. 7 and 8). The mentioned inter- and intra-cell line heterogeneity with regard to colony size, morphology and staining intensity upon different treatments further adds complexity to this problem (Fig. 6). Nevertheless, future technical improvements, including the implementation of machine learning–based solutions, could help to address these issues and will hopefully offer more reliable automated workflows of colony counting, thus potentially outperforming human subjects[66–68].

Data analysis according to power regression (option A) or PE-based normalization (option B)

This protocol provides two mathematical analysis approaches for clonogenic survival data: a generally applicable power regression–based algorithm and a PE-based algorithm for noncooperatively growing cell model systems. Their limitations need to be considered separately.

The generally applicable approach relies on power regression and interpolation (option A). Accordingly, errors originating from the fitting procedure should be minimized to achieve reliable results. This requires an acceptable degree of biological replication ($n \geq 3$). Moreover, a caveat of any regression-based analysis needs to be given attention: an insufficient number of *S*-*C* variable pairs will result in rather erroneous estimations of the fitted parameters (*a* and *b*). We therefore strongly recommend using this workflow with a minimum of five appropriately different *S*-*C* variable pairs per treatment. The goodness of fit should be double-checked for all regressions. Regression coefficients $R^2 \geq 0.9$ are considered acceptable. Uncertainties of the fitted parameters can be used to approximate the uncertainties of the estimated SFs by first-order Taylor series expansion of the formula for SF calculation (e.g., as implemented in the provided MS Excel template file (Supplementary Table 1) and the CFAcoop R-Package (https://cran.r-project.org/web/packages/CFAcoop)).

The PE-based approach (option B) is mathematically less complex and does not rely on regression analysis. However, this algorithm should be applied only in settings without relevant cellular cooperation. This refers not only to the control conditions but also to all treatment conditions of interest. Otherwise, pseudo-precise survival results with underestimated assay-intrinsic systematic errors will be calculated[17,69]. Uncertainties of results obtained by this analysis algorithm can be determined by first-order Taylor approximation (e.g., as implemented in the provided MS Excel template file (Supplementary Table 2)).

For both mathematical approaches, the technical limits with regard to very harsh treatment conditions should be considered. For instance, it may be impossible to obtain individual wells with a sufficient number of colonies after high-dose irradiation of certain cell model systems.

Materials

Reagents

- Cell type(s) of interest (e.g., A-549 cells (RRID: CVCL_0023) from the American Type Culture Collection or EFM-19 (RRID: CVCL_0253) cells from the Deutsche Sammlung von Mikroorganismen und Zellkulturen) **! CAUTION** If established, continuously growing cell lines are used, ensure proper authentication (e.g., by short tandem repeat typing) and ensure that cells are not contaminated with mycoplasma.
- Suitable cell culture media (we use DMEM (1×) + GlutaMAX (Thermo Fisher Scientific, cat. no. 31966-021) for A-549 cells and RPMI 1640 medium (1×) + GlutaMAX + HEPES (Thermo Fisher Scientific, cat. no. 72400-021) for EFM-19 cells)
- Supplements for cell culture medium, such as FCS (e.g., Thermo Fisher Scientific, cat. no. 10270-106), penicillin-streptomycin (e.g., Thermo Fisher Scientific, cat. no. 11548876), and others. We have found that medium supplementation with 10% (vol/vol) FCS and 1% (vol/vol) penicillin-streptomycin mix are required for both A-549 and EFM-19 cells. **▲ CRITICAL** Note that type, quality and batch of the FCS preparation may have a relevant impact on clonogenic growth and cellular cooperation.
- PBS, pH 7.2 (e.g., Thermo Fisher Scientific, cat. no. 14190250)
- Additional reagents and consumables necessary for preparation of single-cell suspensions (Box 2), cell counting (Box 3) and plating (Box 4)
- 70% (vol/vol) ethanol (e.g., Carl Roth, cat. no. T913) **! CAUTION** Ethanol is flammable. Keep away from sources of ignition.
- Low-gelling-temperature agarose (gel formation at ≤31 °C; e.g., Sigma-Aldrich, cat. no. A9045) **▲ CRITICAL** This is required only if embedding cells for 3D growth in semi-solid medium.

- Methylene blue (e.g., PanReac AppliChem, cat. no. A1402) **! CAUTION** Methylene blue is toxic. Handle with care and always wear protective gloves and goggles. **▲ CRITICAL** This is required only for methylene blue staining of adherent 2D colonies (Step 6A).
- Nitro blue tetrazolium chloride (e.g., Thermo Fisher Scientific, cat. no. N6495) **! CAUTION** Nitro blue tetrazolium chloride is toxic. Handle with care and always wear protective gloves and goggles. **▲ CRITICAL** This is required only for nitro blue tetrazolium chloride staining of embedded 3D colonies (Step 6B).

Equipment

- Six-well culture dishes (e.g., Corning, cat. no. 353046)
- 50-ml polypropylene centrifuge tubes (e.g., Sarstedt, cat. no. 62.547.254)
- 1.5-ml polypropylene microcentrifuge tubes (e.g., Eppendorf, cat. no. 0030121872)
- Stereomicroscope with continuous 8–40× zoom function (e.g., Zeiss Stemi 305, cat. no. 435063-9010-100)
- Hemocytometer (e.g., Neubauer Improved, BLAUBRAND; Brand, cat. no. 717805) or other device for cell counting
- X-ray source (e.g., X-Strahl, RS225) or any other agent(s) of interest to be tested

Software

- MS Excel template files provided with this article: MS Excel template file for power regression–based analysis (Supplementary Table 1) and MS Excel template file for PE-based normalization (Supplementary Table 2)
- R-package CFAcoop (https://cran.r-project.org/web/packages/CFAcoop)
- Any other analysis software capable of linear regression (of log-transformed *S*-*C* value pairs) for parameter estimation and calculation of SFs

Reagent setup

Preparation of six-well plates for adherent 2D growth in liquid medium ● Timing 10 min

1 Using serological pipettes, add a sufficient amount of culture medium into the six-well plates (e.g., 2 ml per well).

2 Place the plates into the incubator to equilibrate (physiological temperature and medium-matched CO_2 concentration) just before use.

Preparation of six-well plates for embedded 3D growth in semi-solid medium ● Timing 2–3 h

1 *Prepare a 4% (wt/vol) agarose stock solution*. Add 4 g of agarose to 100 ml of PBS and allow agarose dissolution by carefully heating and swirling the mixture in a microwave oven. Subsequently autoclave agarose. Cool down to ~45 °C and optionally compensate for osmolarity changes resulting from evaporation by adding autoclaved deionized water. **! CAUTION** Avoid superheating of agarose solution and wear protective goggles.

2 Prepare a 37 °C water bath or heating block in or directly next to a laminar flow cabinet for aseptic cell culture to store 4% (wt/vol) agarose and culture medium at 37 °C. **▲ CRITICAL STEP** This heating device is required to keep agarose solutions at optimal temperature. Lower temperatures will cause premature solidification of agarose.

3 Dilute the 4% (wt/vol) agarose solution 1 + 4 with culture medium to generate a 0.8% (wt/vol) agarose solution in a prewarmed 50-ml tube. Mix gently and keep at 37 °C.

4 Using serological pipettes, add 2 ml of the 0.8% (wt/vol) gel mixture into each well of the six-well dishes, avoiding formation of air bubbles. Work quickly to avoid premature agarose gelling. Please see Troubleshooting.

5 Distribute the liquid agarose layer evenly by moving the six-well plates several times in the x-y direction.

6 Allow agarose to gel in six-well plates for 10–20 min at 4 °C on a balanced, flat surface and store until further use. Plates can be stored over night at 4 °C (prevent from drying out). Please see Troubleshooting.

7 Maintain 4% (wt/vol) agarose stock solution at 37 °C for cell embedding (Box 4).

0.8% (wt/vol) methylene blue solution

Transfer 8 g of methylene blue powder into a glass flask containing 1 liter of 70% (vol/vol) ethanol at room temperature (20 °C). Close the flask firmly, agitate vigorously and allow full dissolution of methylene blue on a magnetic stirrer. The solution is stable for >6 months at room temperature and

can be reused for staining several times. ▲ CRITICAL This is required only for methylene blue staining of adherent 2D colonies (Step 6A).

0.8% (wt/vol) nitro blue tetrazolium chloride solution

Transfer 0.8 g of nitro blue tetrazolium chloride into a glass flask containing 100 ml of PBS at room temperature. Close the flask firmly and allow full dissolution on a magnetic stirrer. The solution is stable for 2 weeks at 4 °C. ▲ CRITICAL This is required only for nitro blue tetrazolium chloride staining of embedded 3D colonies (Step 6B).

Conditioned media

As discussed in Facilitation of colony growth, conditioned medium can be used to improve growth of colonies for some cell types. Here, we describe how to generate conditioned medium from A-549 lung carcinoma cells (RRID: CVCL_0023). This approach can also be used for other cell lines. All steps should be performed under aseptic conditions.

1 Seed 2.5×10^6 A-549 cells into a T175 culture flask containing 50 ml of culture medium and let them adhere over night.
2 Incubate the cells for a time sufficiently long to allow conditioning of culture medium (e.g., 72 h for A-549 cells).
3 Harvest the culture medium (50 ml) by using a sterile pipette and transfer it to a 50-ml centrifuge tube.
4 Ensure full removal of proliferating cells by spinning down the culture medium (300*g* and 5 min) and transfering 45 ml of the cell-free supernatant to a fresh tube. Optionally, subject the medium to filtration with low-protein-binding sterile filters (0.2 µm; e.g., Merck-Millipore, cat. no. SLGV033RS). We usually do this with A-549 culture supernatants to remove cell debris and remaining cells. Snap-freeze immediately at −80 °C. ▲ CRITICAL STEP Ensure that there are no viable, proliferating cells in the conditioned medium preparation.
5 Prewarm medium and use in place of usual medium before starting the incubation period for colony formation (plating option 3A(vii) and plating option 3B(ix)). Conditioned medium may be used pure or diluted with fresh nonconditioned medium at optimized concentration. We usually use 1 + 1 diluted conditioned medium for adherently growing cell lines, such as A-549 and EFM-19.

Procedure

Pre-experimental procedure and assay optimization

▲ CRITICAL Several clonogenic assay parameters require context-dependent optimization. Therefore, (i) a method for satisfying colony growth in a desired 2D or 3D format, (ii) suitable *S* on control and treated plates, (iii) assay incubation time, (iv) the occurrence of cellular cooperation and (v) the appropriate mathematical analysis approach for downstream survival calculations need to be determined in the pre-experimental procedure as described in Experimental design.

Generation and plating of single-cell suspensions ● Timing **3 h to 1 d, depending on the assay format**

▲ CRITICAL Steps 1–5 should be performed under sterile conditions.

1 Label all six-well plates properly (one or two plates for both controls and representative treatments). At least five wells with different cell densities for controls as well as for any representative treatments should be prepared. Additional plates to optimize other parameters, such as cell adhesion (in 2D assays) and total incubation time, are recommended.
▲ CRITICAL STEP To identify the most convenient assay design, it is necessary to determine whether cellular cooperation occurs in any of the treatment conditions of interest. This can be achieved only by seeding very different cell densities at this stage, even if the PE-based analysis approach is preferred for the main experiment.
2 Make sure that complete culture medium, PBS and all reagents for the generation of a single-cell suspension and cell plating are prewarmed and ready for use.
3 Choose between option A (pretreatment plating) and option B (posttreatment plating) to test cytotoxic agents. If no cytotoxic treatment is required, proceed with Step 3A(i–v) and omit the treatment steps (Step 3A(vi–viii)).

The two workflows diverge with regard to the time point of plating and cytotoxic treatment(s) (Fig. 3). In option A, single-cell suspensions from one untreated donor culture are plated into the

dishes where clonogenic survival is assessed, and cells are treated immediately upon adhesion (2D format) or embedding (3D format), respectively. In option B, the cytotoxic treatment of interest is performed before plating into the assay dishes, thus giving rise to multiple donor cultures. Either immediately afterwards or after a fixed recovery period, independent single-cell suspensions and dilutions thereof are prepared from all treated donor cultures, and posttreatment plating is performed in either 2D adherent or embedded 3D growth format.

For both options, it is recommended to plate serial dilutions of at least five different cell densities per treatment condition (one or two six-well plates per treatment condition), to synchronize incubation periods under nonphysiological conditions for all plates, such as inadequate temperature and insufficient CO_2 concentration, and to keep the latter as short as possible.

(A) **Pretreatment plating**

(i) Prefill six-well plates with culture medium to allow either adherent 2D or embedded 3D colony growth, as detailed in Reagent setup.

(ii) Harvest the cells from a donor cell preparation and generate a (near-perfect) single-cell suspension. Details of different methods to generate single-cell suspensions are described in Box 2.

(iii) Transfer a small aliquot of the cell suspension to a 1.5-ml microcentrifuge tube and determine the cell count as described in Box 3.

(iv) Using fresh 50-ml centrifuge tubes, prepare serial dilutions of the single-cell suspension suitable for the experimental setup, such as 10^5, 10^4, 10^3 and 10^2 cells/ml.
▲ **CRITICAL STEP** The dilutions need to be prepared with great care, and pipetting errors need to be minimized. Ensure that micropipettes are regularly calibrated.

(v) Plate the cells into the six-well plates prefilled with liquid medium (2D growth) or over a bottom layer of semi-solid culture medium (3D growth) as described in Box 4.

(vi) Once single cells have adhered (2D growth) or equilibrated after 3D embedding, simultaneously remove all dishes from the incubator.

(vii) Carefully refresh any liquid culture medium without disturbing the single cells. This step can be omitted if the volume difference between all wells upon seeding is minimal (<5%).

(viii) Treat the cells with any agent(s) of interest. Make sure that the final assay volume is identical in all wells. Examples of treatment options are discussed above.

(B) **Posttreatment plating**

(i) Seed a sufficient number of cells into the desired number of donor culture flasks (one flask for each treatment of interest). If required, incubate in a humidified incubator (physiological temperature and medium-matched CO_2 concentration) until cells are adapted to the culture medium and/or the desired cell density is obtained.

(ii) Treat the donor flasks with any agent(s) of interest.

(iii) Depending on the research question of interest, either incubate the treated cells in a humidified incubator (physiological temperature and medium-matched CO_2 concentration) to allow repair from potentially lethal damage followed by delayed plating (usually 6–24 h) or move directly to the next step to perform immediate plating.

(iv) Prefill six-well plates with culture medium in preparation for either 2D adherent or 3D embedded colony growth. Details are given in Reagent setup.

(v) Harvest the cells from all donor cell preparations and generate (near-perfect) single-cell suspensions. Details of different methods to generate single-cell suspensions are described in Box 2.

(vi) Transfer small aliquots of the cell suspensions to 1.5-ml microcentrifuge tubes and determine the cell counts according to Box 3.

(vii) Using fresh 50-ml centrifuge tubes, prepare serial dilutions of the single-cell suspensions suitable for the experimental setup, such as 10^5, 10^4, 10^3 and 10^2 cells/ml.
▲ **CRITICAL STEP** The dilutions need to be prepared with great care, and pipetting errors must be minimized. Ensure that micropipettes are regularly calibrated.

(viii) Plate the cells into the six-well plates prefilled with liquid medium (2D growth) or over a bottom layer of semi-solid culture medium (3D growth) as described in Box 4.

(ix) Once single cells have adhered (2D growth) or equilibrated after 3D embedding, simultaneously remove all dishes from the incubator. Carefully refresh any liquid culture medium without disturbing the single cells. This step can be omitted if the volume difference between all wells upon seeding is minimal (<5%).

Incubation period, fixation and staining ● Timing **1–5 weeks**

4 Before starting the incubation period, make sure that the final assay volume is identical in all wells. ▲CRITICAL STEP Varying volumes of culture media in different treatment conditions need to be avoided because this may heavily influence clonogenic survival of single cells in a given dish.

5 Place the culture plates into a humidified incubator (physiological temperature and medium-matched CO_2 concentration). Incubate until colonies on both control and treated plates have reached a sufficiently large size by allowing a minimum of six population doublings. Depending on the cell line and the respective proliferation rate, this may take ≤1 week to >1 month. For A-549 and EFM-19 cells, we usually incubate for 14 and 28 d, respectively. Ideally, control and treated plates should yield at least five wells with consecutive and differing seeding cell numbers that have countable numbers of colonies per well. The colonies should be visible by eye and/or with a (stereo-)microscope.
▲CRITICAL STEP Do not move the plates during the incubation period, and avoid opening and closing the incubator within the first days of growth. Use additional plates from Step 1 to identify an ideal incubation time without disturbing other plates.
? TROUBLESHOOTING

6 Remove all culture dishes from the incubator and proceed with a suitable staining procedure. For adherent 2D growth, we recommend that staining be carried out as described in option A by using an ethanolic methylene blue staining solution. Alternatively, other dyes, such as crystal violet can be used[1]. For embedded 3D colonies, staining can be omitted if visibility of unstained colonies is acceptable. If staining of embedded colonies is desired, metabolic dyes, such as nitro blue tetrazolium chloride (option B) can be used[52]. Viable cells convert this water-soluble dye into a dark violet precipitate. This greatly increases the visibility of small cell clusters, but the cellular architecture may be destroyed by the precipitate. Visualization of 3D colonies with the DNA dye DAPI is an alternative for proper analysis of cellularity in individual 3D colonies. However, this is more laborious and requires a fluorescent microscope[55].

(A) **Fixation and staining of adherent 2D colonies** ● Timing **40 min to 1 d, depending on staining time**

(i) Remove the culture medium from the wells either by carefully pouring off or by aspiration. Ensure that the amount of remaining medium is minimal.

(ii) Fix and stain the colonies by carefully adding 2 ml of 0.8% (wt/vol) methylene blue solution. Make sure not to detach the colonies accidentally.
? TROUBLESHOOTING

(iii) Incubate for 0.5–24 h. Sufficient staining of colonies from different cell lines requires varying incubation times. Stain until colonies are clearly detectable. We find that A-549 and EFM-19 cells require 1 h and 0.5 h minimum, respectively. Overstaining is not possible, but the staining solution should not dry out in the wells.

(iv) Remove and optionally recollect the methylene blue solution. Place the lid back onto the culture dishes.

(v) Fill the sink with warm tap water (30 °C) and rinse the six-well plates for 1 min with the lids closed.
? TROUBLESHOOTING

(vi) Remove the plates from the sink and let air-dry at room temperature or in a drying cabinet (60–80 °C).
■PAUSE POINT Stained six-well plates can be stored for years after staining at room temperature if kept dry and protected from light. Restaining of dry plates is possible if desired.

(B) **Staining of embedded 3D colonies** ● Timing **~1 d**

(i) Carefully remove any liquid culture medium on top of the agar layer by aspiration.

(ii) Add 200 µl of 0.8% (wt/vol) nitro blue tetrazolium chloride staining solution to each well and place the dishes into the incubator (physiological temperature and medium-matched CO_2 concentration).

(iii) Incubate for 16–24 h. Sufficient staining of colonies from different cell lines requires varying incubation times. Stain until colonies are well detectable.

(iv) Remove culture dishes from the incubator. Optionally, take high-resolution photographs of all wells at suitable magnification (in z-stacks) and/or proceed with colony counting in the dishes. Avoid dehydration of gel matrices, which occurs if plates are stored for a long time.

Colony counting of pre-experiment and development of final assay design ● Timing 1–1.5 h

7 Count all colonies with ≥50 cells by using a (stereo-)microscope. Depending on cell type and colony size, this is best performed at 8–40× magnification. For each treatment, start with the lowest *S*. Proceed with increasing *S* values. Stop counting colonies for a given treatment as soon as the number of colonies per well is too high for counting (overgrown culture dishes and/or much more than ~200 colonies per well).

▲ **CRITICAL STEP** Staining intensity, shape, cellular morphology and size of colonies with >50 cells may be very different between cell lines as well as within one experiment, especially when harsh treatments are tested (Figs. 6 and 8). In many cases, counting without magnification will give rise to false-positive and/or false-negative scorings. In particular, colonies with very tiny cells should not be overlooked, whereas abnormally large cells or diffuse aggregates must not be scored (Fig. 8). In case of embedded 3D growth, colonies in all layers of the semi-solid medium need to be considered.

? TROUBLESHOOTING

8 For each treatment condition, identify the highest *S* value, where all biological replicates resulted in zero colonies ($C = 0$) and exclude all counting results with identical or lower *S* values from the analysis.

9 Enter the raw data for the pairs of values (*S*, *C*) of all biological replicates in a statistical software tool capable of power regression analysis (i.e., linear regression of log-transformed *S*-*C* data), or use the MS Excel template file provided with this article (Supplementary Table 1). For details, see also Figs. 4, 5, and 9.

10 Average the *C* values from all biological replicates for each individual *S* value.

11 Perform power regression analysis according to $C = a \times S^b$ for the averaged pairs of values (*S*, *C*) and determine the parameters *a* and *b* for untreated controls and all treatment conditions.

? TROUBLESHOOTING

12 Design the final assay setup and choose the mathematical approach (power regression–based or PE-based) to be used in the main experiment. These decisions should rely on wells with reasonable colony counts of all dishes (Step 7) and the *b* values obtained from power regression analysis (Step 11). Composition of culture medium, 2D or 3D assay format, generation of single-cell suspensions, plating according to option A or option B, ideal numbers of seeded cells and adherence and incubation parameters should now be set. Harsh treatments with overall poor colony growth should be excluded from the main experiment.

▲ **CRITICAL STEP** Note that the power regression–based approach counterbalances the influence of cellular cooperation. For each treatment of interest, it requires at least five to six countable wells covering the range of ideally <10 to >100 colonies per well. The PE-based approach may appear more convenient, because fewer cell densities need to be plated, and survival calculations are less complex. However, its use is strongly discouraged in conditions of nonlinear relationships between *S* and *C* as reflected by *b* values ≥1.2.

Main experiment ● Timing 1–5 weeks

13 Use the final assay parameters as optimized in Step 12 and perform your experiment in three or more independent biological replicates by repeating Steps 1–7 with the chosen assay setup (power regression–based or PE-based analysis), the resulting cell densities needed and the optimized parameters as described above.

14 Proceed with clonogenic survival data analysis by using either power regression–based analysis (option A) or PE-based normalization (option B).

(A) **Data analysis of the final experiment by using power regression ● Timing 30 min**

(i) Determine power regression parameters *a* and *b* as described in Steps 8–11 for control and all treatment conditions by using a statistical software tool, the MS Excel template file provided with this article (Supplementary Table 1) or the CFAcoop R-Package (https://cran.r-project.org/web/packages/CFAcoop).

? TROUBLESHOOTING

(ii) Using the determined parameters *a* and *b*, calculate the *S* values needed to expect $C = 20$ colonies for controls and all treatment conditions.

(iii) Determine the SFs for all treatment conditions by calculating the $SF_{treated} = S_{untreated}/S_{treated}$ ratios at $C = 20$. Uncertainties of the calculated $SF_{treated}$ values can be approximated from the variance-covariance matrix of the regression parameters via error propagation by

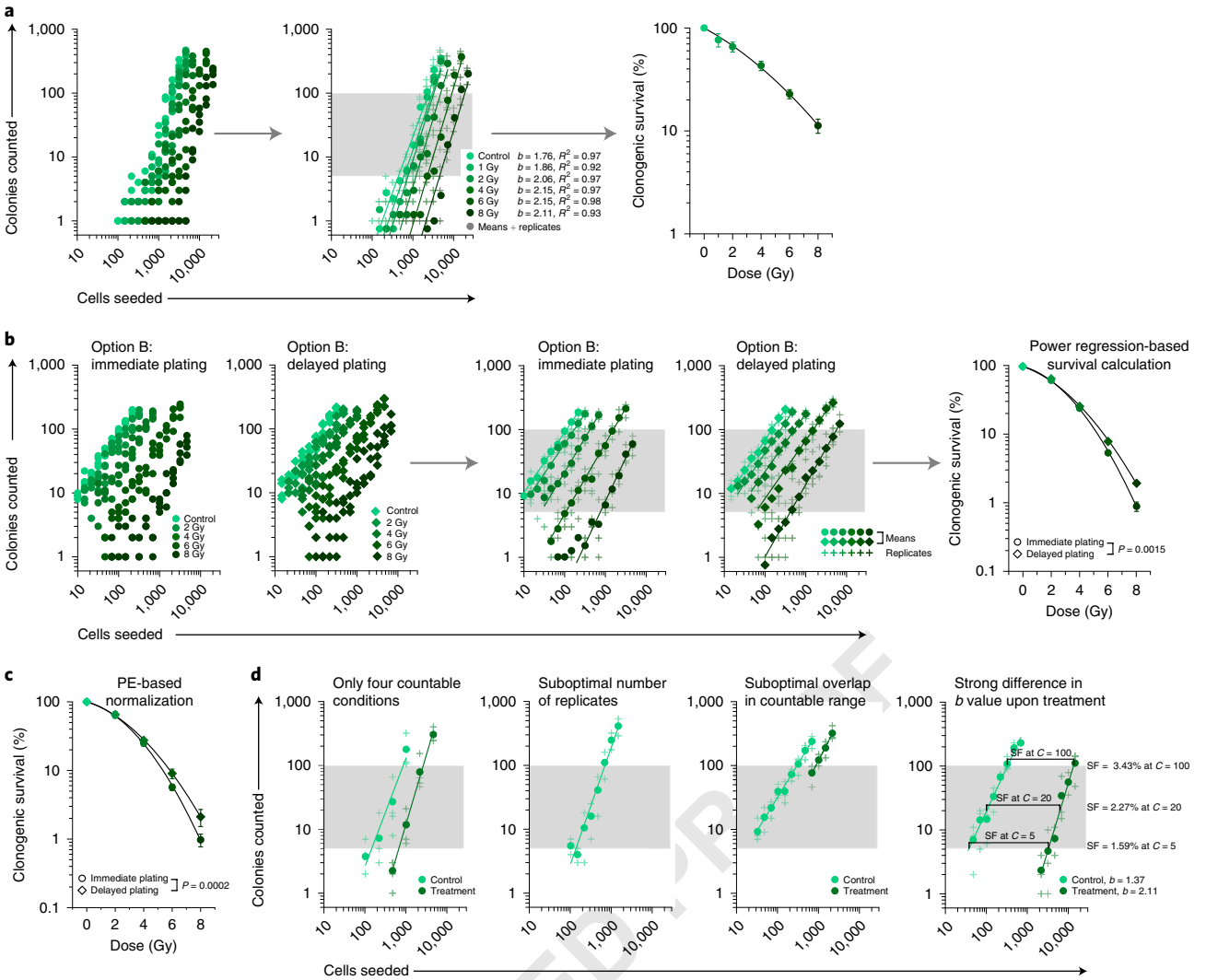


Fig. 9 | Examples of clonogenic survival data analysis. a, The breast cancer cell line BT-20 (RRID: CVCL_0178) was plated according to option A and treated with 0–8 Gy (four biological replicates; data taken from Brix et al. with slight modifications[17]). Data were subjected to power regression according to $C = a \times \mathbf{S}^{\mathbf{b}}$, and **b** values of each fit and the corresponding regression coefficients R^2 were determined. The survival curve derived from this dataset (SF values obtained for $C = 20$ with corresponding standard deviations as determined by first-order Taylor approximation, plotted in dependence of the radiation dose with superimposed linear-quadratic fitting) is displayed in the right panel. **b**, Clonogenic survival of the glioblastoma cell line A172 (RRID: CVCL_0131) upon irradiation at 0–8 Gy was tested by using plating option B with both immediate and delayed plating variants (three biological replicates each). Power regression and calculation of SFs for both plating variants were performed as in **a** (left), and mean SF values ± s.d.s are shown. Statistical comparison was performed by ANOVA of nested models (*F* statistics: $F = 49.2$, degrees of freedom ($df1 = 2$, $df2 = 4$, $P = 0.0015$). **c**, The same dataset as in **b** was subjected to PE-based normalization, yielding nearly identical results. Statistical comparison of immediate and delayed plating was performed by ANOVA of nested models (*F* statistics: $F = 144.2$, $df1 = 2$, $df2 = 4$, $P = 0.0002$). **d**, Examples of experiments requiring optimization simulated with hypothetical data. Power regression was impaired by insufficient numbers of *S*-*C* value pairs (first panel) or insufficient numbers of biological replicates (second panel). SF value calculation was suboptimal if the range in the countable overlap of *S*-*C* value pairs was small (third panel). An assay-intrinsic error originated from largely different *b* values across treatments (fourth panel). Here, the *b* values of control and treatment differed strongly, and the uncertainties of calculated SF values were rather large, because SF values increased systematically with increasing *C* values.

using first-order Taylor series expansion of $SF_{treated}$ (e.g., as implemented in the provided MS Excel template file (Supplementary Table 1) or the CFAcoop R-Package (https://cran.r-project.org/web/packages/CFAcoop)).

(B) **Data analysis of the final experiment by using PE-based normalization** ● Timing **30 min**

(i) Determine for each individual biological replicate the average PE from all control wells by using a statistical software tool or the MS Excel template file (Supplementary Table 2).

? TROUBLESHOOTING

(ii) Calculate the SFs for each biological replicate at all treatment conditions according to:

$$SF_{treated} = \frac{C_{treated}/S_{treated}}{\mathrm{PE}}$$

Uncertainties of $SF_{treated}$ values can be approximated from the variances of the $C_{treated}/S_{treated}$ ratios and PE estimates via error propagation by using first-order Taylor series expansion (e.g., as implemented in the provided MS Excel template file (Supplementary Table 2)).

(iii) Determine the mean SFs for each treatment condition by averaging the $SF_{treated}$ values of the biological replicates. Uncertainties of the mean SFs can be estimated from the standard deviations of the corresponding biological replicates. A lower bound of this uncertainty can be obtained via the sum of the $SF_{treated}$ variances of the corresponding biological replicates (e.g., as implemented in the provided MS Excel template file (Supplementary Table 2)).

? TROUBLESHOOTING

15 Plot the SFs versus any treatment conditions. For experiments testing ionizing radiation, a semi-logarithmic presentation is conventionally used (*x* axis: radiation dose; *y* axis: log(survival)), and survival data are commonly subjected to linear-quadratic fitting[70]. Alternative approaches, including nonlinear logistic fitting, dimensionality reduction and unsupervised hierarchical clustering, may also be helpful[71].

16 (Optional) For statistical comparison of different survival curves (e.g., between two cell lines), several options may be used. If the dose-response relationship is fitted according to an established model (e.g., linear-quadratic fitting in the case of clonogenic survival upon ionizing radiation), the SFs can be subjected to ANOVA comparison of nested models (*F*-statistics). If no underlying regression model is used, two-way ANOVA (without interaction term) can be used.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible reason	Solution
5	All or many wells of one or more treatment option(s) are either empty or (sub-)confluent	Inappropriate range of cell numbers seeded	Adjust the range of cell densities to be tested and/or plate additional cell densities Increase or decrease the incubation time Consider changing growth conditions (e.g., media and supplements), the assay format (2D vs 3D growth) and/or composition of 3D matrix
	Fewer than five consecutive wells with a countable number of colonies	Inappropriate range of cell numbers seeded	Adjust the range of cell densities to be tested and/or plate additional cell densities Increase or decrease the incubation time Consider changing growth conditions (e.g., media and supplements), the assay format (2D vs 3D growth) and/or composition of 3D matrix
	Hardly any cell growth	Inappropriate culture conditions for single cells: medium-derived growth factors; auto- and paracrine stimulation of cell growth are insufficient to sustain cell growth	Consider the use of other culture media, alternative additives and/or measures to facilitate colony formation as described in Facilitation of colony growth. Consider using alternative assay formats (2D or 3D)
6A(ii and v)	2D colonies detach during the staining procedure	Too harsh treatment; colonies with highly epithelial morphology can detach easily	Avoid pipetting directly onto the colonies, add staining solution cautiously and rinse the plates with great care
7	Irregular colony growth; poor correlation between number of cells seeded and colonies counted	Suboptimal growth conditions for a fraction of the wells of one experiment	Avoid using culture media from different batches within one biological replicate of the assay. Growth conditions can be different between two supposedly identical medium preparations Do not move the plates during the incubation; avoid repeated opening and closing of the incubator

Table continued

Table 2 (continued)

Step	Problem	Possible reason	Solution
11 and 14A(i)	The dataset cannot be subjected to power regression according to $C = a \times S^b$	Power regression is only doable if the dataset to be fitted does not contain any zero values for averaged *C* values	Check whether power regression was performed with *S* values where *C* equals zero in all biological replicates
	Flattening increase of colony numbers at increasing cell densities	Scoring of overgrown wells	Check whether high-density wells were inappropriately counted in Step 7. If necessary, exclude them from analysis
14B(i and iii)	Determined PE values vary strongly with cell density seeded, and uncertainties of calculated SFs are huge	PE-based normalization is not appropriate for the respective setting	Repeat the assay with power regression–based analysis
Box 2, options A(vi) and B(iii)	Cell aggregates instead of single cells	Suboptimal cell dissociation protocol	Test alternative detachment/dissociation reagents and incubation times and/or carefully intensify mechanical separation of cell clumps with micropipettes. Use cell strainers combined with (repeated) washing steps in PBS
Box 3, step 3	The suspension leaks out of the chamber	Improper preparation of the chamber	Rinse the chamber in 70% (vol/vol) ethanol, let the chamber dry and repeat the filling procedure
Box 3, step 4	<20 or >250 cells per 1-mm^2 square	Dilution not suitable for manual cell counting[72]	Prepare appropriate dilutions of the single-cell suspension
	Poor cell viability after generating the single-cell suspension (high percentage of trypan blue–positive cells)	Too harsh enzymatic and/or mechanical treatment	Handle cells with greater care when generating the single-cell suspension, decrease enzyme incubation time and/or use other enzyme(s)
			In the case of cells cultured in vitro, use donor cultures with lower cell density to facilitate dissociation
Box 4, option A(iv)	Cells do not adhere to culture dishes	Too harsh treatment or too long incubation of cells in single-cell suspension under nonphysiological conditions (suboptimal temperature and insufficient CO_2 concentration)	Generate and handle the single-cell suspension with greater care
			Reduce incubation times under nonphysiological conditions
		Suboptimal conditions for adhesion	Do not move the plates during the adhesion process; avoid repeated opening and closing of the incubator
Box 4, option B(ii)	Matrix gels prematurely	Unsuitable agarose preparation	Choose an agarose type with melting temperature clearly <37 °C
			Use prewarmed serological pipettes and/or increase room temperature (>20 °C)
Box 4, option B(iv)	Matrix does not gel	Too low agarose concentration and/or suboptimal gelling temperature	Allow gelling at lower temperature but avoid cold-shocking the cells
			Slightly increase the concentration of the gel-forming matrix (e.g., 1% agarose for the bottom layer and 0.5% for embedded cells)

Timing

The timing estimates given here assume that the researcher has basic knowledge in aseptic mammalian cell culture techniques. The total hands-on time of a single-replicate clonogenic experiment (e.g., 10 six-well plates for the assay optimization procedure and 10 six-well plates for the main experiment) is usually <20 h. Depending on the chosen assay format, it varies considerably. The total time required is largely determined by the proliferation rate of the cell model system of interest and may be as long as 10 weeks (up to 5 weeks for assay optimization as well as the main experiment).

Cell model system-dependent assay optimization

Steps 1–3, generation and plating of single-cell suspensions: 3 h to 1 d, depending on the cell model system of interest and the chosen assay format (origin of cells, procedure to generate single-cell suspension, 2D or 3D growth and treatment options)

Steps 4–6, incubation period and staining: 1–5 weeks, depending on the doubling time of cells

Steps 7–12, colony counting of the pre-experiment and development of the final assay design: 1–1.5 h

Main experiment

Step 13, main experiment: 1–5 weeks, depending on optimized assay format
Step 14, final data analysis: 30 min for an experiment with three to four biological replicates
Steps 15 (plotting of results) and 16 (optional; statistical comparison of treatments): 30 min

Anticipated results

Figure 9a shows the raw data of the colony-counting results of one representative, successful experiment performed with the pretreatment plating procedure. The human breast cancer cell line BT-20 (RRID: CVCL_0178) was treated with radiation doses of 0–8 Gy (four independent biological replicates; data taken from ref. [17]). Variable pairs of S and C with $C = 0$ for all replicates were excluded from the analysis as described in the data analysis section of the protocol (Step 8). Power regression and SF value calculation were accomplished according to Step 14A(i–iii), and data were further processed according to Step 15 (Fig. 9a). Obtained b values ranged from 1.76 to 2.15, implying that the chosen cell line exhibits a very high degree of cellular cooperation with an approximately quadratic relationship between S and C. R^2 values were consistently ≥0.9, indicating that power regression in this experiment worked well. The determined uncertainties for SF values calculated at $C = 20$ at a given treatment dose according to first-order Taylor approximation were small. For comparison with conventional, PE-based normalization of survival results of the identical dataset, see Fig. 2 in ref. [17].

In principle, power regression can be performed as simple linear regression of log-transformed data or by minimizing some distance of the regression curve to the original data. In addition, regression can be performed on the values of individual replicates or after averaging over replicates. The different approaches give rise to sets of regression lines that slightly differ in steepness and diverge in accounting for individual biological replicates with $C = 0$. Importantly, the final SF values obtained across all experiments that we have analyzed so far were only marginally affected by these differences. Nevertheless, on the basis of our experience, we recommend performing power regression in terms of linear regression on log-transformed values upon averaging over replicates, because linear regression on log-transformed values represents a well-established model with defined error term and because regression on averaged values can include S-C value pairs with $C = 0$ in individual replicates and contains less uncertainty in the fitting results.

A172 (RRID: CVCL_0131), an established human glioblastoma cell line, was plated by using option B (posttreatment plating) (Fig. 9b). Two survival curves either upon immediate plating or upon additional incubation for 24 h before plating (delayed plating), respectively, were obtained by the presented analysis workflow. Distinct differences in clonogenic survival were observed, and linear-quadratic regression curves are superimposed. For statistical comparison, the SFs (at $C = 20$) were subjected to ANOVA of nested models (F statistics: $F = 49.2$, degrees of freedom (df)$1 = 2$, $df2 = 4$, $P = 0.0015$). Because in this experiment, the observed degree of cooperation was very low (b values ≈ 1.0), the PE-based algorithm can also be implied for data analysis and yields highly similar results, yet with larger standard deviations (F statistics: $F = 144.2$, $df1 = 2$, $df2 = 4$, $P = 0.0002$) (Fig. 9c).

Figure 9d exemplifies hypothetical results of experiments that need optimization. Power regression is impaired if the range or the overall number of countable S and C variable pairs are too small and/or the number of independent biological replicates is too low. Furthermore, the comparison of different treatment conditions is compromised if the overlap in the countable ranges is too limited and calculation of SF values occurs in the extrapolated range beyond data acquisition. Finally, differences in b values emerging from different treatment conditions render the extraction of SF values challenging, because SF values show systematic tendencies to increase or decrease with increasing or decreasing C values, respectively. This is not a specific problem of the analysis algorithm presented here but rather a general limitation of the clonogenic assay itself, which exacerbates with PE-based normalization (see Fig. 2 in ref. [17]).

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All colony-counting raw data of clonogenic survival experiments in this article (i.e., S-C value pairs of all biological replicates) are provided in the Source Data for Figs. 5 and 9. Some of the clonogenic survival data displayed in Figs. 5 and 9 were taken from ref. [17], as noted in the corresponding figure

legends. Schematic graphs in Figs. 2, 4 and 9d were generated from hypothetical datasets. All other data supporting the findings of this study are available within the article and its supplementary information files. Additional information can be provided by the corresponding author upon request. Source data are provided with this paper.

Code availability

The paper is accompanied by two MS Excel template files for the presented analysis workflows: Supplementary Table 1 for the power regression–based analysis approach and Supplementary Table 2 for PE-based normalization. The R-package CFAcoop is available at https://cran.r-project.org/web/packages/CFAcoop.

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Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft DFG (SFB1321, Project-ID 329628492, P16), the Bundesministerium fuer Bildung und Forschung BMBF (ZiSStrans NUK047A, NUK047C, METABOLiST NUK061C and DKTK) and the International graduate program iTarget (Elitenetzwerk Bayern).

Author contributions

N.B. and K.L. conceived and designed the protocol with support from D.S., H.Z. and C.B. N.B. acquired the data. D.S. provided mathematical consultation and developed and programmed the R-package CFAcoop. The MS Excel template files for power regression–based and PE-based survival analysis were generated by N.B. and D.S. N.B. and K.L. wrote the manuscript, and all authors commented on and discussed the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-021-00615-0.

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Peer review information *Nature Protocols* thanks Tatsuya Ohno, Daniel Wahl and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Received: 23 December 2020; Accepted: 10 August 2021;

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Colony counting and selection of representative colonies for images was performed with a stereomicroscope (Stemi 305 Series, Carl Zeiss, Jena, Germany) at 8-40-fold magnification. Photographs were taken using either the Labscope Software (Version 3.0.1, Carl Zeiss, Jena, Germany), an Axio Lab A1 microscope equipped with an AxioCam ERc 5s (Carl Zeiss) or with a Samsung Galaxy A71 smartphone camera (Samsung, Seoul, South Korea).
Data analysis	OriginPro 2021 and MS-Excel were used for regression analyses, statistical procedures, and interpolations.

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All colony counting raw data of clonogenic survival experiments in this article (i.e. S-C value pairs of all biological replicates) are provided in Source Data Figure 5 and Source Data Figure 9. The authors declare that some of the clonogenic survival data displayed in Figure 5 and Figure 9 of this manuscript were taken from Brix et al. 17 as specified in the corresponding figure legends. All other data supporting the findings of this study are available within the article and its supplementary information files. Additional information can be provided by the corresponding author upon request.

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Sample size	No statistic method was used to predetermine sample size. The sample sizes in our experiments were determined based on experience and commonly accepted standards in the field.
Data exclusions	Data points at low densities were excluded if all biological replicates of an experiment yielded zero colonies in order to allow logarithmic transformation and regression. Culture dishes with exceedinly high numbers of colonies were not considered for counting.
Replication	All experiments were performed in three to four independent biological replicates. Information on data replication is provided in the respective Figure Legends.
Randomization	Cell culture of varying density and passage number (passage 2-10 post thawing) were randomly used for experiments.
Blinding	No blinding of investigators was performed.

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Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	All cell lines were obtained from ATCC (Manassas VA, USA), the DSMZ (Braunschweig, Germany), or CLS (Heidelberg, Germany), respectively.
Authentication	Authentication of the frozen stock aliquots was performed by STR profiling at the cell authentication service from the DSMZ.
Mycoplasma contamination	All cell lines were routinely tested negative for Mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.