LDAcoop: Integrating non-linear population dynamics into the analysis of clonogenic growth in vitro

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Abstract

The limiting dilution assay (LDA) is a well-established technique to determine the frequency of clonogenic cells with self-renewing capacity in mammalian cell populations in vitro. Data analysis is commonly performed by using linear, single-hit Poisson mathematics. However, clonogenic growth under single-cell conditions frequently exhibits cooperative or competitive population dynamics, thus violating the linearity assumption of existing LDA analysis tools. Here, we present a novel mathematical modeling approach that integrates non-linear dynamics into LDA analysis. We developed LDAcoop - an R-based computational tool that can be universally employed to quantify clonogenic cells in limiting dilution setups. Using multiple cell types, we benchmarked the LDA format against the similarly well-established colony formation assay (CFA). We demonstrate that LDA outperforms CFA particularly in settings with patient-derived organoids, suspension cells, and applications with higher throughput requirements. Collectively, these properties predestine the LDA format and the new analysis tool LDAcoop for complex cell culture models, larger-scale screenings, and advanced automated analyses of clonogenic growth experiments.

Introduction

The quantification of clonogenic mammalian cells *in vitro* represents a key method in the toolbox of researchers from a variety of life science disciplines, including cell biology, stem cell research, immunology, toxicology, and oncology. In this context, clonogenic growth describes the capacity of single cells to form colonies through self-renewal and division. Clusters with a minimum of 50 cells which developed from one singular ancestor cell are conventionally considered as a surrogate for infinite growth or – at least – the capacity for multiple rounds of cell division, respectively (Brix et al., 2021; Rafehi et al., 2011).

A widely used technique to determine the frequency of mammalian cells with self-renewing, clonogenic potential is the limiting dilution assay (LDA) (Hu and Smyth, 2009). Serial dilutions of single-cell suspensions are seeded into multi-well plates, and the fraction of wells with clonogenic growth for each seeded single-cell number is determined (**Figure 1**). The frequency of clonogenically active cells in the single-cell population can be inferred from the failure fraction, i.e. the fraction of wells without clonogenic growth, according to the zero term of the Poisson distribution at an expectation of one (Hu and Smyth, 2009) (**Figure 2A**). A similarly well-established technique to quantify clonogenic growth is the colony formation assay (CFA) (Brix et al., 2021; Franken et al., 2006). Here, the exact colony count per culture dish in relation to the number of seeded cells is determined (Brix et al., 2021). Interestingly, the use of LDA and CFA is very unevenly distributed across different research disciplines. While the use of LDA clearly dominates in research publications within hematology, cell tissue engineering, virology, and immunology, CFA is notably overrepresented in toxicology, radiology, and food science technology. (**Supplemental Figure 1A**). In oncology, both methods find application in a roughly balanced manner. Nevertheless, oncological studies focused on cancer stem cell biology preferentially use LDA protocols, whereas CFA protocols are more commonly applied in studies examining combination treatments and dose-response relationships (**Supplemental Figure 1B**). So, it appears that there is a notable discrepancy in the prevalence of utilization between LDA and CFA formats for the quantification of clonogenic cells – both, between distinct life science disciplines and within the large field of oncological research.

As a mutual characteristic, the LDA and the CFA interrogate clonogenic growth behavior at very low densities, often less than one cell per mm^2 surface area. Under these extreme conditions, colony formation is critically dependent on growth-supporting soluble factors in the culture medium, and it was not until the 1950s that reproducible mammalian single-cell growth became technically feasible (Puck and Marcus, 1955; Sanford et al., 1948). At this time, scientists had a thorough understanding of the *in vitro* growth requirements for various types of mammalian normal and cancerous cells, and clonogenic single-cell growth was known to be dependent on biochemically defined molecules – salts, amino acids, sugars, and cofactors – as well as a complex and undefined mixture of animal-derived

essential components typically contained in serum preparations (Eagle, 1955; Puck, 1958). Although clonogenic growth of selected cell lines became feasible in fully synthetic, chemically defined media in the 1960s (Ham, 1965), serum preparations or other animal-derived supplements, such as bovine pituitary extract, are still widely used in contemporary cell culture (Yao and Asayama, 2017).

In addition to the biochemical composition of the culture medium itself, cell-derived molecules, such as growth factors, hormones, low-molecular weight metabolites, and growth inhibitors, can influence single-cell growth *via* auto- and paracrine mechanisms (Sporn and Todaro, 1980), suggesting that phenomena of cellular cooperation and competition influence clonogenic growth patterns to varying extents (Archetti and Pienta, 2019). In this context, it has been proposed that the absence of cooperating or competing neighboring cells can respectively slow down, inhibit, or enhance cell growth at low plating densities (Gerlee et al., 2022; Hershey et al., 2023; Johnson et al., 2019; Pomp et al., 1996; Takizawa et al., 1993). The reduction in reproductive fitness at limiting cell densities is known as the Allee effect – a concept in ecology to describe a *per capita* population growth rate which gradually decreases at low population densities (Johnson et al., 2019; Stephens and Sutherland, 1999). On the contrary, neighboring cells can also exert competitive effects under limiting cell numbers, *via* deprivation of critical nutrients in the chosen culture medium and/or secretion of growth-inhibitory factors (Takizawa et al., 1993). Hence, albeit less common than cellular cooperation, competitive mechanisms may contribute to non-linear clonogenic growth phenomena as well (Pomp et al., 1996; Takizawa et al., 1993; van Neerven and Vermeulen, 2023; Yao and Asayama, 2017), and the net effect of cooperative and competitive effects in a culture dish obviously depends on various parameters, including the cell culture model and the culture conditions chosen.

Cellular cooperation and competition strongly affect the mathematical analysis of clonogenic survival experiments. However, standard data analysis workflows and associated computational tools fail to account for these non-linear phenomena, causing the linear mathematical models to diverge from the real-world data they are meant to represent (Brix et al., 2020). To address this limitation in the CFA, we recently developed a mathematical modeling approach that incorporates non-linear cell number to clonogenic growth relations and thereby improves analytical reliability and robustness (Brix et al., 2021; Brix et al., 2020). In the present study, we investigated the effects of non-linear clonogenicity in the LDA format. Considering that clonogenic survival analyses in both the CFA and LDA format are performed using very different dilutions of single-cell suspensions within one experiment (Brix et al., 2021; Hu and Smyth, 2009), we hypothesized that cooperative as well as competitive effects may similarly lead to unreliable estimates of clonogenic cell frequencies in the LDA format due to violations of the linearity assumption in stable ratios of cell numbers to measures of successful clonogenic growth. Accordingly, we generalized the established linear, single-hit Poisson model *via* power law and included a non-linearity parameter which accounts for cooperative and competitive clonogenic growth

phenomena. To make this workflow accessible for a broad audience, we developed LDAcoop, an R package published on CRAN (accompanied by an online shiny app version thereof) designed to account for non-linear clonogenic growth patterns in LDA analysis. Finally, we benchmarked the LDA format against the CFA format with regard to experimental procedures, scoring, data output, speed, and versatility in different cancer cell culture systems.

Results

Non-linear phenomena of clonogenicity: Cellular cooperation and competition and their impact on data analysis in limiting dilution assays

To assess the impact of non-linear phenomena on the robustness of clonogenicity analyses in the LDA format, we measured clonogenic survival upon irradiation in a panel of adherent cancer cell lines with varying degrees of cooperative and competitive clonogenic growth behavior as previously demonstrated in the CFA format (Brix et al., 2020). For the given cell line panel, we found – as expected – that some cell lines showed non-linear relations between the number of cells seeded (*S*) and the log-transformed fraction of wells with clonogenic growth failure (*ln(μ)*) (**Figure 2B**). This indicates that the single-hit Poisson model, which describes a scenario where one event (i.e. clonogenic growth of one cell) occurs randomly and independently at a constant average frequency, and which is typically assumed in standard LDA analyses (Agro and O'Brien, 2015; Hu and Smyth, 2009; Ploemacher et al., 1989; Strijbosch et al., 1987; Taswell, 1981) is not universally applicable. To investigate this phenomenon in greater detail, we plated fixed numbers of A549 and SKLU1 single-cells into multiple wells with highly different volumes of culture medium (20–5000 µl/well, 384-well–12-well format) and analyzed clonogenic growth. In line with the approximately linear *ln(μ)~S* relation of A549 cells, the clonogenic activity of these cells was quite stable in different culture medium volumes when plated in standard medium supplemented with 10% FCS, and the majority of wells (> 80%) revealed clonogenic growth (**Figure 2C,** right graph). In contrast, SKLU1 cells showed a high degree of cellular cooperation and, correspondingly, a steep decline in clonogenic growth with increasing assay volume, thus providing a demonstrative explanation for the non-linear *ln(μ)~S* relation of this cell line (**Figure 2C,** left graph). These data confirm previous results showing that potentially clonogenic cells may "lose" their clonogenicity if cellular cooperation *via* soluble factors is hampered (Brix et al., 2020). As expected, highly variable estimates of the fraction of clonogenic cells ranging from 1/8 to 1/175 cells were obtained when applying the (obviously violated) LDA single-hit model to SKLU1 cells in different culture medium volumes.

Vice versa, A549 single-cells grew highly competitively when clonogenicity was assessed under serum-reduced conditions, and frequency estimates of clonogenic cells covered the full range from 1/1 to 1/inf when analyzing the raw data with linear *ln(μ)~S* regression (**Figure 2D,** right graph). Volume-

independent growth patterns were obtained for SKLU1 cells if EMEM, the basal medium recommended by the supplier, was replaced by DMEM/F12, a more nutrient-rich medium supplemented with the identical percentage of 10% FCS (**Figure 2D,** left graph). Taken together, these data demonstrate that clonogenic growth of single cells plated at limiting cell numbers is strongly affected by cell density and the composition of the culture medium which together frame the biochemical context for single-cell growth. Individual cells with – in principle – clonogenic potential may fail to give rise to clonogenic offspring under the limiting dilution conditions of the LDA format, whereas they may manage to survive clonogenically when supported by others, or when using alternative culture media containing all factors required for clonogenic growth at a sufficient concentration. Conversely, competition for growth factors and/or nutrients may impair clonogenic survival.

LDAcoop: Implementing non-linear population dynamics into LDA data analysis

These deviations from linear clonogenic growth behavior have been described by various researchers (Adrian et al., 2018; Brix et al., 2021; Brix et al., 2020; Eliason et al., 1985; Hershey et al., 2023; Pomp et al., 1996; Veldwijk et al., 2014), and cellular cooperation (Archetti and Pienta, 2019) as well as cellular competition (van Neerven and Vermeulen, 2023) of mammalian cells provide biological explanations for violations of the single-hit hypothesis of LDAs in these cell model systems. In order to account for distorting effects of non-linear clonogenicity on the analysis of CFA-derived survival data, we recently proposed a regression-based mathematical analysis workflow CFAcoop, where the relation of clonogenic cells and seeded cells is modeled via power law (Brix et al., 2021; Brix et al., 2020). We now aimed to address this issue in the mathematical analysis workflow of LDA experiments. Commonly, the fraction of clonogenic cells (p) in a given cell population is calculated by determining the total number of cells ($S_{\lambda=1}$) that need to be plated in order to obtain on average exactly one clonogenic cell ($\lambda = 1$), i.e. exactly one colony, per well. According to the Poisson distribution, this mean of exactly one colony per well corresponds to a probability of clonogenic growth failure (i.e. no colony per well) in $1/e$ of all wells tested, which is approximately 37%. The average of clonogenic cells obviously depends on (i) an independent probability p of clonogenic growth for each individual cell and (ii) the total number S of cells seeded. In standard linear, single-hit Poisson models (SHPM), the expected number of clonogenic cells is described by

$$\lambda_{SHPM} = p \cdot S.$$

To account for non-linear (i.e. cooperative or competitive) clonogenic growth phenomena, analogously to CFAcoop we generalized this relation *via* power law and included a non-linearity parameter b to

$$\lambda = p \cdot S^b.$$

Inserting this non-linear relation into the failure term of the Poisson distribution (for the number of clonogenic cells in a well) yields

$$P_\lambda(k = 0) = e^{-p \cdot S^b}.$$

Accordingly, the number of cells required for an expectation of one clonogenically active cell per well and their corresponding cooperativity parameter *b* can be determined on the basis of the fraction of wells with clonogenic growth failure. From the fractions of clonogenic cells under control and treatment conditions, the surviving fractions (*SFs*) can be inferred. A detailed description of the mathematical background and the development of the R package LDAcoop is given in the Materials and Methods section, and a step-by-step example of LDA raw data collection, scoring as well as plotting of multiple replicate data, subsequent non-linear *ln(μ)~S* fitting, and the final clonogenic survival results is shown in **Figure 3**.

In full analogy to our CFAcoop approach for CFA analysis (Brix et al., 2021; Brix et al., 2020), the exponent *b* is determined independently for each treatment condition of an experiment by assessing the probability of clonogenic growth over a range of different cell numbers seeded in *n* replicate wells. In case of cellular cooperation, *b*-values of > 1 are observed, whereas cellular competition is characterized by *b*-values < 1. Within the cell line panel shown in **Figure 2B**, we found clear deviations from the linearity assumption ($b \approx 1$) in both directions with *b*-values ranging from 0.70 to 0.92 for T47D cells and from 1.54 to 2.60 for SKLU1 cells at different radiation doses which were in good agreement with the results obtained in the CFA format (**Supplemental Figure 2** and (Brix et al., 2020)).

The implementation of the exponent *b* into the analysis workflow stabilizes the results output and increases its robustness (**Figure 4**). The estimated clonogenic activity of a given cell population with non-linear clonogenicity as well as the corresponding confidence intervals remain very stable, irrespective if the full available data set or reduced versions thereof (data set reduced at the higher or lower range of seeded cells) are used for calculation. In strong contrast, utilizing the linear analysis approach (Hu and Smyth, 2009) yields highly varying results for the estimated clonogenic activity, including the corresponding confidence intervals. Hence, power law-based modeling of non-linear clonogenic growth phenomena as implemented in LDAcoop clearly outperforms LDA analysis tools that rely on linear equations in conditions with violated linearity assumption. While the original authors explicitly discouraged application outside the method's valid scope (Hu and Smyth, 2009), such cases remain common in practice.

Benchmarking LDA and CFA: LDA is superior for complex culture models and applications with higher throughput requirements

Considering the virtually identical readouts of CFA and LDA – both are used to determine the fraction of clonogenic cells after various treatments – we set out to compare the results generated by the two methods. Clonogenic survival curves for the seven cell lines were calculated by using LDAcoop, and the results were compared to previously generated CFAcoop results from cells of the same cultures (difference of no more than 3 culture passages between data collection) (Brix et al., 2020). The survival curves of the seven cell lines were highly similar (**Figure 5A**). Obviously, both CFA and LDA can be used equivalently to assess clonogenic survival, provided that non-linear clonogenic growth behavior is properly considered.

Given the congruence of CFA and LDA results outputs, we next benchmarked the two methodologies against each other with regard to time and costs. Six independent researchers with different levels of experience in clonogenic assay analysis were asked to score identical sets of CFA and LDA plates with each six different treatment conditions. While highly similar survival results were obtained for the CFA format, researchers with less experimental experience tended to underscore the LDA format, particularly in the lower dose range of treatment (**Figure 5B**). However, the time required for assay scoring was significantly shorter for the LDA format – irrespective of the researchers' level of experience – (**Figure 5C**), translating into a total reduction of time needed for an entire LDA experiment compared to the CFA format. The costs for consumables of both methods were comparable (**Figure 5D**).

Considering the advantages of clonogenic survival analyses in the LDA format in 2D settings with adherent cells, the performance of LDAcoop was tested in cell culture models for which CFA protocols are highly challenging and time-consuming to be employed. We used the suspension breast cancer cell line DU4475 which is not able to form adherent colonies in liquid culture medium (Langlois et al., 1979) and analyzed its survival upon irradiation by LDA. While the determination of the precise colony count/well in liquid culture media would obviously be impossible and thus require more labor-intensive surrogate analyses in semisolid 3D matrices, LDA scoring was well feasible even without fixation and staining of the cells (**Figure 6A, B**). With *b*-values of up to 3.75, cellular cooperation in DU4475 cells was profound, thus confirming the necessity to consider this phenomenon in LDA analysis.

We also examined clonogenic survival upon irradiation in three different patient-derived organoid cell lines which were cultured in 3D matrices covered by liquid culture medium (**Figure 6C**). Organoid formation by irradiated single cells seeded in the LDA format could be obtained in individual wells for

all conditions tested, and viability of organoids was confirmed in selected culture wells by immunofluorescence microscopy (**Figure 6D, E**). These data show that the LDA format can be easily adapted to challenging cell culture models, whereas the necessity to determine the precise number of colonies in individual wells frequently obstructs the applicability of the CFA format, particularly if non-adherent cells or organoid culture systems are analyzed.

Finally, we made use of 384-well culture dishes to interrogate the upscaling potential of the LDA format. Yes/no scoring of culture plates was well feasible, and the increase in information density as compared to 96-well dishes of identical size was 4-fold (**Figure 7A**). Additionally, increasing the number of technical replicates *n* (**Figure 7B**) per cell density as well as increasing the number of informative dilutions *d* (**Figure 7C**) can be instrumentalized to generate more reliable results with smaller confidence intervals. Hence, these experiments demonstrate the potential for larger-scale screening approaches in the LDA format which would be much more challenging in the CFA format.

Discussion

In the present study, we developed a novel mathematical modeling approach that integrates non-linear phenomena of clonogenicity into LDA analysis. We introduce LDAcoop – a computational LDA analysis tool that accounts for cooperative and competitive clonogenic growth behavior in analogy to CFAcoop in which power law-based analysis of CFA data captures non-linear clonogenicity (Brix et al., 2021). LDAcoop is provided in an R package version (https://cran.r-project.org/web/packages/LDAcoop/index.html) as well as in an online shiny app format (https://helmholtz-munich-zyto.shinyapps.io/LDAcoop/).

The clonogenic capacity of single cells can be modulated by soluble factors which are fully absent or present at limiting concentrations in the culture medium. In case of cellular cooperation, higher single-cell densities result in elevated concentrations of cell-derived factors, finally leading to increased clonogenicity (Brix et al., 2021; Brix et al., 2020). *Vice versa*, cellular competition for growth-limiting nutrients and/or growth factors or driven by growth-inhibiting factors may occur, respectively (Takizawa et al., 1993). In consequence, the pattern of auto- and/or paracrine growth modulation of single cells is shaped by (i) the secretome of the cells (ii) the biochemical composition of the culture medium, (iii) the kinetics of metabolite–receptor interactions, and (iv) the biophysical properties of the culture system (Brix et al., 2021; Brix et al., 2020; Shvartsman et al., 2001). This also implies that a mammalian cell's clonogenic capacity is not an inherent yes/no characteristic but is rather contingent upon the permissiveness of the chosen culture conditions.

Cell density effects under limiting dilution conditions have been extensively characterized *in vitro*. Historically, mammalian single-cell growth experiments were experienced to be very challenging, because unconditioned, cell-derived growth factor-free culture media were recurrently observed to be insufficient for sustaining single-cell growth (Puck and Marcus, 1955; Sanford et al., 1948), thus giving rise to the concept of "autocrine secretion" by Sporn and Todaro (Sporn and Todaro, 1980). More recently, we and others demonstrated that soluble cell-derived factors enforce clonogenic survival and proliferation rates even when using modern, advanced culture media (Brix et al., 2020; Hershey et al., 2023; Lim et al., 2013). Mathematical modeling as well as experimental evidence suggest that autocrine growth stimulation may even be relevant in scenarios where only one singular cell is seeded into a culture dish (Shvartsman et al., 2001). We conclude that a strict binary categorization of individual cells of a given population into clonogenic versus non-clonogenic cells is a misleading oversimplification. It can distort the calculation of clonogenic cell frequencies, because even cells with high clonogenic potential may show impaired clonogenicity or complete abrogation of their clonogenic capacity, respectively, if the cells' culture requirements are insufficiently addressed. Along the same lines, abrupt deprivation of individual cells from cooperating neighbors has been proposed as explanation for low plating efficiencies in clonogenic *in vitro* experiments, slow recovery of cell lines when passaging them at limiting cell densities, and poor success rates in the establishment of stably growing tumor cell lines from *ex vivo* preparations and clinical tumor specimens (Axelrod et al., 2006), all of which can be interpreted as examples of the Allee effect of cell populations growing under low-densities *in vitro* (Gerlee et al., 2022; Johnson et al., 2019).

Because it is technically impossible to find "ideal" culture conditions which fully compensate for cooperative and competitive effects in clonogenic survival experiments for each cell model system and each treatment condition of interest, we recently proposed a mathematical approach that accounts for these phenomena in downstream CFA data analysis (Brix et al., 2021; Brix et al., 2020). In CFAcoop, the relation between the number of single cells seeded (*S*) and the number of colonies obtained (*C*) is fitted by power law according to $C = a \times S^b$ (Brix et al., 2021; Brix et al., 2020). In the present study, we transfered this approach to the analysis of clonogenic survival in the LDA format and developed the R package LDAcoop. In full analogy to CFAcoop, the degree of non-linear clonogenicity in LDAcoop is represented by the exponent *b* which is mathematically equivalent to the exponent used in CFAcoop (Brix et al., 2021). LDAcoop allows non-linear relations between *S* and $ln(\mu)$, which we indeed observed in various cell lines as indicated by *b*-values ranging from 0.7 (in case of moderate cellular competition) to 3.75 (for extreme cellular cooperation). In line with this, alternative non-linear LDA quantification approaches have already been proposed (Bonnefoix and Sotto, 1994; Ellison et al., 2005). Nevertheless, linear models to date clearly dominate routine LDA analysis (Hu and Smyth, 2009).

Against this background, we now present a mathematical generalization of the most widely used linear model, which additionally captures the non-linear cooperative and competitive dynamics.

Of note, LDAcoop differs from linear LDA analysis tools with regard to the mathematical information that is inferred from experiments where all-negative responses occur for one or more cell densities: For LDAcoop, an all-negative response resulting from seeding each *S* cells into *n* wells is not equivalent to seeding each *N* cells into *s* wells with the same all-negative result, because the dilution of cell-derived factors as well as the total amount of medium-derived factors per cell differ between the two settings if $S \neq n$. In contrast, linear, single-hit models ignore the impact of initial single-cell density and calculate identical estimates of clonogenic cell frequencies in these two scenarios, because a constant total number of cells, $S \times n$, were analyzed (Hu and Smyth, 2009). The association of parameters *p* and *b* implies that LDAcoop does not provide estimates of clonogenic cell frequency if not a single well exhibits clonogenic growth at a given treatment condition. We therefore recommend testing geometric series of single-cell density dilutions to ideally obtain response patterns that cover the full range from all-positive to all-negative for each treatment condition of interest. In contrast, multiple cell densities with all-negative or all-positive responses add little information to the overall dose-response curve.

Apart from the LDA methodology, CFA protocols are routinely employed to quantify clonogenic growth of mammalian cells *in vitro*. In the present study, we show that the two methodologies are complementary and almost exclusively used in distinct research fields of life sciences. Most importantly, we observed that LDA and CFA formats generate highly similar survival data, provided that the impact of non-linear cooperative and competitive clonogenic growth phenomena are properly integrated into the mathematical analysis of raw data (Archetti and Pienta, 2019; van Neerven and Vermeulen, 2023).

Since we demonstrated the equivalence of LDA and CFA results in various cell lines, we additionally benchmarked the two methodologies against each other and found that LDA protocols outperform CFA analyses under certain conditions. This is most obvious in settings where suspension cells or organoids are analyzed. Here, the requirement of the CFA to determine an exact colony count in a 3D matrix complicates the analysis, whereas binary scoring of the LDA format is much more rapid and also feasible for suspension cells without embedding. Hence, researchers from scientific disciplines which so far predominantly rely on CFA approaches may consider the LDA as a time-saving alternative that is well-accepted for the analysis of clonogenic single-cell growth.

A major aspect in the design of LDA experiments is to find an adequate number of technical replicates for each cell density. Most experiments performed in the present study, rely on technical replication

of n = 12 and d = 8 different single-cell dilutions tested per treatment, i.e. 96 wells for each treatment condition. More robust results with reduced uncertainties can be obtained by increasing the number of controls which serve for data normalization (e.g. by using 2 × 96 wells for the untreated controls). Additionally, both higher-resolution data sets with reduced uncertainties at the costs of higher technical replication (e.g. n = 48, d = 8) or, alternatively, lower-resolution data sets with multiple treatments and/or different cell lines at the costs of larger uncertainties (e.g. n = 8, d = 8 for six different cell lines seeded on one plate) can be acquired with a fixed number of total wells. Irrespective of the desired assay format, 384-well plates allow experimental upscaling as compared to 96-well plates, since the density of information per surface area is increased by 4-fold. Albeit limited by a maximal culture medium volume of 10–12 µl per well, which might escalate the impact of cellular competition at rather low cell densities, even automated LDA analysis on 1536-well plates could be of interest for specific research questions, including large-scale screenings of therapy resistance and/or drug response relationships, with the observation endpoint being clonogenic survival.

Cooperation and competition mediated by soluble cell-derived factors that we now implemented into LDA data analysis should not be considered as artificial *in vitro* effects of mammalian cell culture. Although beyond the scope of this study, there is clear evidence that both phenomena occur *in vivo*. For instance, soluble factors such as Decapentaplegic (DPP) in drosophila wings (Moreno et al., 2002) or Wingless/Integrated (WNT) antagonists in murine intestinal crypts (Flanagan et al., 2021) are prominent examples of cellular competition mediated by soluble factors. *Vice versa*, cooperating subclones of established tumors have been characterized (Cleary et al., 2014; Martin-Pardillos et al., 2019; Tabassum and Polyak, 2015). Further evidence for cooperative growth of cancer cells *in vivo* – albeit not necessarily *via* soluble cell-derived factors alone – stems from metastatic seeding experiments. Various groups have shown that distant metastases are frequently formed by small clusters of malignant cells, whereas equal numbers of single cells often revealed lower metastatic potential (Cheung and Ewald, 2016; Cheung et al., 2016; Hu et al., 2020; Liotta et al., 1976). Considering that the LDA format is in principle also applicable *in vivo*, the use of LDAcoop may therefore not be limited to *in vitro* cell culture experiments.

In conclusion, our study presents a novel mathematical workflow that enhances the analysis of clonogenic survival data acquired in the LDA format by incorporating non-linear, cooperative and competitive growth phenomena and introduces LDAcoop, an R package including an online shiny app version thereof. In view of the high congruence of clonogenic survival results obtained by CFA and LDA protocols, the superiority of the LDA format in terms of scoring speed, applicability in suspension and 3D organoid culture settings, as well as its upscaling potential position LDAcoop as a valuable tool for larger-scale screenings and automated analyses of therapy resistance and drug response with the

observation endpoint clonogenic survival, thus resolving significant bottlenecks in research fields which currently rely predominantly on the CFA format.

Materials and Methods

Development of LDAcoop

Statistical modeling of non-linear clonogenic growth behavior

Let *n* be a number of wells with an identical number of cells. The probability of failure (no growth) in each well is identical and independent for all wells and denoted with μ. The number of failures *Y* is therefore a binomially distributed random variable:

$$P(Y = r) = \binom{n}{r} \mu^r (1-\mu)^{n-r}.$$

The probability of success $(1-\mu)$, in turn, depends for each well on the number of cells seeded *S*. Assuming an identical and independent probability of growth *p* for every cell seeded, we find as known from the single-hit Poisson model (SHPM) an expectation of

$$\lambda_{SHPM} = p \cdot S$$

"active" cells in a well. As a generalization of this linear approach and analogously to modeling non-linear growth behavior in CFA analysis, the deviation from linearity in mean activity is accounted for by introducing a non-linearity coefficient *b* yielding a power law relation

$$\lambda = p \cdot S^b.$$

Given a mean activity λ, the number of active cells in a well *X* follows approximately the Poisson distribution

$$P_\lambda(X = k) = \frac{\lambda^k}{k!} e^{-\lambda}.$$

For $\lambda = 1$, the expectation that on average one colony per well is growing, the probability of detecting failure equals the probability of detecting exactly 1 colony in an individual well.

Being interested in the number of cells that need to be seeded to achieve $\lambda = 1$, the fraction of wells with exactly 1 colony (which equals the fraction of failures) can be linked to the fraction of active cells through

$$\mu = P_{\lambda=1}(k = 0) = e^{-p \cdot S^b}$$

we find (with $\alpha = \ln(p)$)

$$\ln(-\ln(\mu)) = \alpha + b \cdot \ln(S).$$

For *m* sets of wells with varying numbers of cells S_i and corresponding observations $\mu_i, i \in \{1, \ldots, m\}$ this is a generalized linear regression model of the binomial family with log-log link function. Model fitting is conducted using statistical software R (*glm* function from *stats* package).

Definition of clonogenic activity

We define the clonogenic activity *a* as the number of cells that need to be seeded to achieve in average 1 colony per well, resulting in e^{-1} (i.e. approximately 37%) wells with exactly 1 colony and e^{-1} wells with growth failure:

$$a = e^{\frac{-\alpha}{b}} = (p^{-1})^{\frac{1}{b}}.$$

For cells with linear clonogenicity, the clonogenic activity equals the inverse of the active cell frequency (Hu and Smyth, 2009). A cell frequency suggests an easy and straightforward interpretation "each cell has a certain probability of having some property", and this probability is the same for all cells, independently of the others. However, the chance of forming a new colony also depends on the surroundings, particularly the number of neighboring cells and their secretomes. Against this background, quantification through an active cell frequency is misleading in the context of cellular cooperation or competition, and the definition of clonogenic activity focusses directly on this total number of cells, which provides together the activity of interest.

The uncertainty of the clonogenic activity is calculated from the confidence bands of the model fit with statistical software (R statistical software, *predict.glm* function from basic *stats* package). Cell numbers that result in upper or lower bounds of 37% negative wells are the lower and upper bounds of the confidence interval of the clonogenic activity (**Supplemental Figure 3**). Alternatively, the uncertainty can be approximated *via* conventional error propagation (e.g. through First-order Taylor series approximation) in terms of standard deviation.

Calculation of surviving fractions

Changes in the clonogenic activity under treatment (e.g. upon exposure to ionizing radiation) are quantified using the survival fraction *SF*. This fraction is defined as the ratio of numbers of cells required to result in an identical expectation of the same reference activity (e.g. 37% failure rate) without and with treatment.

Let α_0 and b_0 denote the model parameters fitted to the untreated cells, and α_t and b_t the parameters for the treated cells. The survival fractions are calculated as

$$SF_t = \frac{a_0}{a_t} = \frac{e^{\frac{-\alpha_0}{b_0}}}{e^{\frac{-\alpha_t}{b_t}}} = e^{\frac{\alpha_t}{b_t} - \frac{\alpha_0}{b_0}}.$$

Uncertainties of the survival fractions can be approximated *via* conventional error propagation. Highly non-linear cell lines require higher orders in Taylor series for approximation. We therefore estimate the uncertainty of survival fractions by combining 83.5% confidence intervals of the corresponding clonogenic activities (Austin and Hux, 2002; Knol et al., 2011; Payton et al., 2003).

Therefore, given a reference clonogenic activity of a_0 with associated 83.5% confidence interval $[a_{0,lb}, a_{0,ub}]$ and a clonogenic activity a_t of a treated group with associated confidence interval $[a_{t,lb}, a_{t,ub}]$, the 95% uncertainty interval of the survival fraction is approximated as $[\frac{a_{0,lb}}{a_{t,ub}}, \frac{a_{0,ub}}{a_{t,lb}}]$ (**Supplemental Figure 3**).

Utilization of biological replicates

Individual biological replicates vary in their characteristics. Thus, measurements from the same biological replicate are not fully independent. Still, there is no unique model including this variability, but multiple options exist how this or that aspect of inter-replicate variability could be modeled. Therefore, to assess inter-replicate variability, we strongly recommend indicating individual biological replicates in plots of the clonogenic activity. This offers direct and easy visual inspection of the magnitude of inter-replicate variability.

In case the inter-replicate variability is considered rather small with respect to its intrinsic stochasticity, combining the number of wells before modeling is reasonable (i.e. numbers of wells from biological replicates are added up). The intrinsic stochasticity stems from the binomial characteristic of the number of responding wells. As an example, the 95% expectation range for 12 wells with probability of nonresponding p = 1/3 is 1 to 7 nonresponding wells (which on *ln(μ)*-scale corresponds to a range of -2.5 to -0.5).

In case inter-replicate variability is considered high and serious underestimation of the calculated uncertainties are expected, all replicates are to be analyzed separately. This means, clonogenic activities and survival fractions are calculated for the replicates separately. In that case, overall clonogenic activities and associated uncertainties can be derived from the sets of individual clonogenic activities and survival fractions by calculating the mean and standard error of the mean.

In case of low numbers of biological replicates combined with extreme high numbers of wells per biological replicate, the calculated uncertainties will also be underestimated (as in the case of high inter-replicate variability). In this case, the experiments should be separated by replicate as well.

Statistical methods

All calculations were performed in R version 4.2.0 (2022-04-22; R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2022). LDA data was analyzed by CRAN LDAcoop package (https://cran.r-project.org/web/packages/LDAcoop/index.html). A shiny app version of LDAcoop is available under https://helmholtz-munich-zyto.shinyapps.io/LDAcoop/. For comparison, in **Figure 2B** and **Figure 4** LDA data (number of cells per well, number of wells, number of positive wells) were also fitted with the "ELDA" function of the "statmod" R package (Hu and Smyth, 2009).

Literature analysis of CFA and LDA use in *in vitro* studies in biomedical sciences

The most-cited research papers for the CFA format (Franken et al. (Franken et al., 2006) 2,329 citations (Web of Science 2023-12) and the LDA format (Hu and Smyth, (Hu and Smyth, 2009) 1,098 citations (Web of Science 2023-12)) were analyzed. Lists of all research papers citing these manuscripts were compiled using Web of Science (Clarivate). The citing literature was subsequently analyzed by research fields, including oncology, cell biology, immunology, and others ("Web of Science Categories"). To assess the use of CFA and LDA formats in the field of oncology, all citing papers were sorted in descending order by their number of citations, and the top 100 cited publications per assay format were used. Review articles, papers that re-used CFA or LDA data from previously published manuscripts, and publications citing either of the two papers to exclusively analyze *in vivo* data were excluded from the analysis. All data figures, tables and supplemental material of these manuscripts were reviewed manually for the search terms indicated. Raw data included in **Supplemental Figure 1A** and **B** are derived from Clarivate Web of Science.

2D cell culture

The breast cancer cell lines T47D, SKBR3, BT20, MDA-MB231, HCC1806, and DU4475, and the lung cancer cell lines A549 and SKLU1 were purchased from either ATCC (Manassas, VA, USA), CLS (Heidelberg, Germany) or DSMZ (Braunschweig, Germany). Cells were cultivated in a humidified atmosphere at 37°C in culture media (all from Thermo Fisher Scientific, Schwerte, Germany) supplemented with fetal calf serum (FCS), 100 U/mL penicillin and 0.1 mg/mL streptomycin. Details are listed in **Supplemental Table 1**. Cell line authentication was performed *via* short tandem repeat typing (service provided by DSMZ), and cell cultures were routinely screened to be free from mycoplasma contamination (MycoAlert Detection Kit, Lonza, Basel, Switzerland).

Organoid cell culture

In agreement with ethical approval granted by the medical faculty of LMU Munich, organoid cell cultures were established from tumor samples of pancreatic ductal adenocarcinoma (PDAC) and head and neck squamous cell carcinoma (HNSCC) as previously described (Mahajan et al., 2021; Weber et al., 2022). The details of medium composition are given in **Supplemental Tables 2 and 3**. All organoids were passaged weekly, PDAC organoids by mechanical dissociation and HNSCC organoids by enzymatic dissociation using TrypLE™ Express (Thermo Fisher Scientific) and subsequent use of the trypsin inhibitor from *Glycine max.* (Sigma-Aldrich, Taufkirchen, Germany). Upon dissociation, the rho-associated protein kinase (ROCK) inhibitor Y-27632 (Selleckchem, Munich, Germany) was added at 10 µM for 1 week (PDAC), or 1 day (HNSCC), respectively.

X-Ray treatment

Irradiation was performed using an RS225 X-ray tube (X-Strahl, Camberley, UK) operated at 200 kV and 10 mA with an integrated Thoraeus filter (1 Gy in 242 s).

Analysis of clonogenic survival of adherent cell lines by LDA and CFA

For the analysis of clonogenic survival of adherent cells in the LDA format, geometric dilution series of single-cell suspensions were seeded into 96-well plates (2^{-3}–2^{12} cells per well). Upon adherence, the medium was refreshed (200 µl per well), and cells were irradiated with the indicated doses. Clonogenic growth was allowed for 14 to 21 days, cells were fixed and stained using 80% ethanol containing 0.8% methylene blue (Sigma Aldrich). Plates were screened for clonogenic growth at 8–40 × magnification. Wells containing at least one colony with ≥ 50 cells were considered positive. Surviving fractions were calculated using the provided R tool LDAcoop.

CFA format data and power regression-based results (i.e. the fraction of clonogenic cells per well after a given treatment) were taken from Brix et.al. (Brix et al., 2020).

Limiting Dilution Assay with suspension cells

Geometric dilutions of DU4475 single-cell suspensions were seeded into 96-well plates (2^{0}–2^{13} cells per well) and the total volume per well was adjusted to 200 µl in all wells. Then, cells were irradiated and incubated for 23 days. Screening was performed at 40 × magnification without prior staining, and surviving fractions were obtained as described for adherent cells.

Limiting Dilution Assay with organoids

Single-cell suspensions of organoids were generated using a mixture of TrypLE™ Express (Thermo Fisher Scientific), Dispase (2 mg/mL) and DNAse I (10 µL/mL) (both Sigma-Aldrich) for PDAC organoids

and TrypLE™ Express alone for HNSCC organoids. The trypsin inhibitor from *Glycine max.* was used to stop the reaction. Single-cell suspensions were diluted 1 + 1 in growth factor-reduced matrigel (Corning, Wiesbaden, Germany) for PDAC cells or BME (R&D Systems, Wiesbaden, Germany) for HNSCC cells, respectively. 10µl of the cell suspension was immediately seeded into 96-well U-bottom plates in a range of 1–3,200 cells per well. After 20 min, culture medium containing 10 µM ROCK Inhibitor Y-27632 was added to the wells. Subsequently, cells were irradiated and incubated for 21 days. Analysis was performed as described for suspension cells.

Fluorescence microscopy of organoids

For fluorescence microscopy, single-cell suspensions of one PDAC organoid line were seeded into eight-chamber slides (Ibidi, Gräfelfing, Germany) and irradiated at the indicated doses. After 21 days, medium was removed and matrigel drops were covered with fresh medium containing 10 µg/mL Hoechst 33342 and 20 µg/mL propidium iodide (both from Sigma-Aldrich). Organoids were imaged after an incubation time of 1 h at room temperature with an AxioObserver Z1 inverted microscope equipped with an EC Plan-Neofluar 5x/0.16 Ph1 and an AxioCam MRm camera.

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Competing Interests

All authors declare that financial, personal, or professional interests do not exist.

Abbreviations

C: colony count per well, CFA: colony formation assay, DPP: decapentaplegic, F: failure fraction, FCS: fetal calf serum, HNSCC: head and neck squamous cell carcinoma, LDA: limiting dilution assay, OR: odds ratio, PDAC: pancreatic ductal adenocarcinoma, ROCK: Rho-associated protein kinase, S: number of seeded cells per well, SFs: surviving fractions, SHPM: single-hit Poisson model, WNT: wingless/integrated

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Figure Legends

Figure 1: The limiting dilution assay (LDA).
A) Principle of the LDA. A dilution series of the single-cell suspension of interest (with cell numbers S_1 to S_8) is seeded into a multi-well plate with multiple replicates per dilution. After an appropriate incubation time sufficient to allow at least 5 cell doublings, the number of wells with clonogenic growth failure is determined, and clonogenic activity is calculated via the zero term of the Poisson distribution with $\lambda = 1$. **B)** Experimental workflow as performed in this manuscript. **C)** Examples of raw data from MCF7 cells. LDA plates were seeded in an 8-step geometric dilution series with 12 replicates per dilution. The numbers of seeded cells (S) and the fractions of wells with clonogenic growth failure ($\mu = Y/n$) are indicated.

Figure 2: Linear single-hit models do not account for cooperative and competitive clonogenicity and fail to robustly describe clonogenic growth behavior of several cell lines in the LDA format.
A) Workflow of LDA data analysis with existing tools relying on linear, single-hit Poisson mathematics (such as https://bioinf.wehi.edu.au/software/elda/ (Hu and Smyth, 2009)). **B)** Examples of LDA raw data sets of seven different cancer cell lines with linear regression superimposed in red (95% confidence intervals are displayed in pale red). Data of each cell line were generated in three to four independent biological replicates. Mean $ln(\mu)$ values are represented by filled dots (•). + and ∇ symbols represent data points from individual replicates. **C)** Analysis of mammalian single-cell growth behavior types. Identical cell numbers of SKLU1 and A549 single-cell suspensions (10 cells/well for SKLU1, 3 cells/well for A549) were seeded into 384-well, 96-well, 24-well and 12-well plates in culture medium volumes of 20–5000 µl per well, and the percentage of wells with clonogenic growth was determined after 21 days for SKLU1 and 14 days for A549 cells. Data of four independent biological replicates each performed in 48–96 technical replicates are given, and power regression lines with 95% confidence intervals are superimposed. **D)** Identical cell numbers of SKLU1 and A459 cells were seeded as in B) in varying culture medium volumes of DMEM/F12 medium supplemented with 10% FCS (left panel) or varying concentrations of FCS (right panel). Data of four independent biological replicates each performed in 48–96 technical replicates are given, and regression lines (power or logistic regressions) with 95% confidence intervals are superimposed.

Figure 3: Overview of the LDA data analysis workflow with the R tool LDAcoop. A) Layout of a representative 96-well plate with a geometric dilution series of 8 different cell numbers (S) seeded in 12 technical replicates. The fraction of wells with clonogenic growth and without clonogenic growth (failure wells) is determined for each cell number. **B)** Raw data of failure fractions (μ) of different cell numbers are plotted in an $ln(\mu)$~S graph. μ-values are averaged, generalized linear regression models are fitted to the $ln(\mu)$~S data for controls and each treatment dose to determine the clonogenic activities (S-values corresponding to the failure rates $\mu = 1/e$), and the respective 95% confidence bands are displayed in pale green. Clonogenic survival curves are generated by normalizing the clonogenic activity values of each treatment dose to that of the untreated controls. Data (of BT20 cells) were collected in four independent biological replicates each performed in 12 technical replicates.

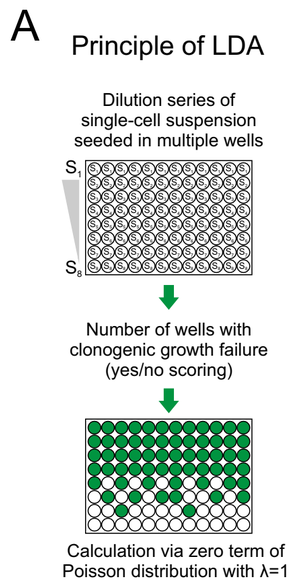
Figure 4: Implementation of a non-linearity coefficient b strongly increases robustness of fit. A) Fits with linear $ln(\mu)$~S (Hu and Smyth, 2009) and non-linear $ln(\mu)$~S^b regression (LDAcoop) of the full data set of BT20 cells shown in Figure 2B (8 Gy), or reduced versions thereof with omission of data points in the higher (left panel) or lower (right panel) cell number range. Regression lines and confidence intervals are shown. **B)** Estimated clonogenic activities and their confidence intervals as calculated in A.

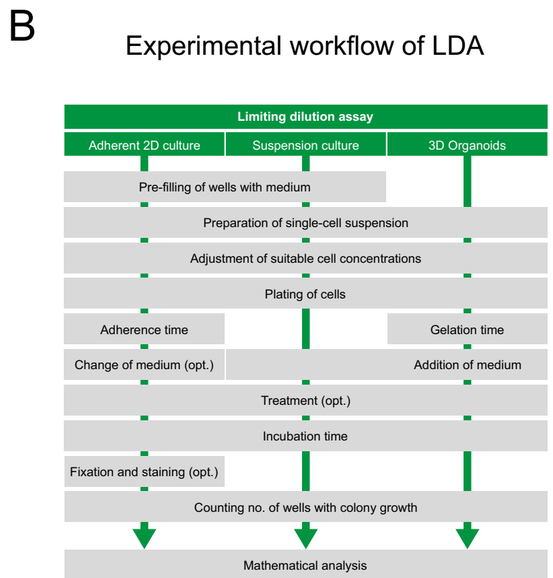
Figure 5: Clonogenic survival results generated by CFA and LDA protocols are highly similar, but the LDA format requires less time for scoring. A) Comparison of the survival curves of seven cancer cell lines generated by CFA and LDA protocols. CFA data were measured in three (T47D and HCC1806 cells)

or four (all other cell lines) independent biological replicates, subjected to analysis and CFAcoop, and taken from Brix et al. (Brix et al., 2020). LDA data were acquired in three (SKBR3 and A549 cells) or four (all other cell lines) independent biological replicates, and clonogenic survival curves were calculated using the R tool LDAcoop. **B)** Comparison of inter-researcher variability in the CFA and LDA format. Survival data of each one CFA and one LDA experimental replicate of SKBR3 cells were scored by six independent researchers with varying extents of experience and subjected to downstream analysis with CFAcoop or LDAcoop, respectively. **C)** Time required for scoring the data shown in A). **D)** Comparison of total experimentation time and costs of CFA and LDA.

Figure 6: Clonogenic survival analyses of non-adherently growing cell lines and organoids using the LDA format. A) Analysis of clonogenic survival upon irradiation by LDAcoop using the non-adherently growing breast cancer cell line DU4475. Means and 95% confidence intervals of three independent biological replicates are shown. **B)** Photographs of untreated (left) and irradiated (right) DU4475 cells on the day of analysis (d21 after plating). One representative well of a 96-well plate is shown in the large photograph (surface area = 0.32 cm^2). Inlays depict colony growth at higher magnification. **C)** Scheme depicting clonogenic organoid formation in the 96-well format. **D)** Analysis of treatment response of patient-derived organoids by LDA. Single-cell suspensions of two pancreatic cancer organoids (PancO1, PancO2) and one organoid from a head and neck squamous cell carcinoma (HNSCC) were irradiated at 0–8 Gy and clonogenic organoid formation was analyzed by LDAcoop. Means and 95% confidence intervals boundaries of three independent biological replicates are shown. Transmitted light and fluorescence microscopy images of representative pancreatic cancer organoids on the day of analysis (d21 after plating and irradiation at the indicated dose). Live-dead staining of matrigel-embedded organoids was performed with Hoechst 33342 and propidium iodide.

Figure 7: Upscaling potential of the LDA protocol to the 384-well format and optimization of assay design. A) Layout of 384-well plates to analyze the impact of varying numbers of technical replicates *n* (left panel) or varying numbers of informative cell dilutions *d* (right panel) on the robustness of results output. Photographs of HCC1806 cells grown in the respective 384-well formats. **B)** Analysis of clonogenic activity, clonogenic survival, and the corresponding uncertainties (width of confidence interval) of the setup with varying numbers of technical replicates *n* as shown in A). **C)** Analysis of clonogenic activity, clonogenic survival, and the corresponding uncertainties (width of confidence interval) of the setup with varying numbers of informative cell dilutions *d* as shown in A).


A
Principle of LDA
Dilution series of single-cell suspension seeded in multiple wells
S1
S8
Number of wells with clonogenic growth failure (yes/no scoring)
Calculation via zero term of Poisson distribution with λ=1


B
Experimental workflow of LDA
Limiting dilution assay
Adherent 2D culture
Suspension culture
3D Organoids
Pre-filling of wells with medium
Preparation of single-cell suspension
Adjustment of suitable cell concentrations
Plating of cells
Adherence time
Gelation time
Change of medium (opt.)
Addition of medium
Treatment (opt.)
Incubation time
Fixation and staining (opt.)
Counting no. of wells with colony growth
Mathematical analysis

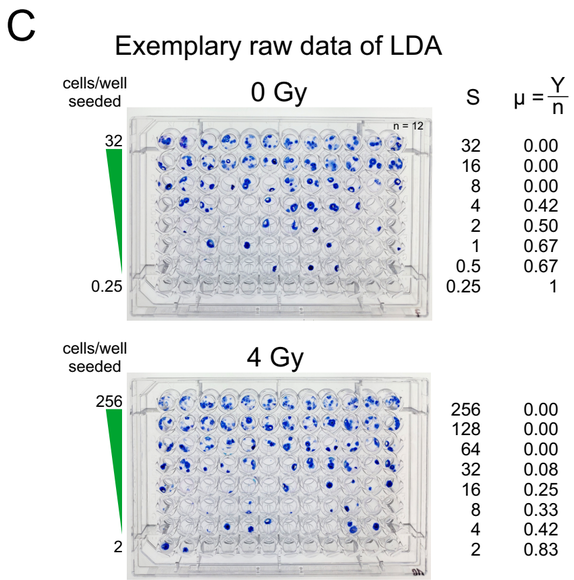

C
Exemplary raw data of LDA
cells/well seeded
0 Gy
n = 12
32
0.25
S
μ = Y/n
32 0.00
16 0.00
8 0.00
4 0.42
2 0.50
1 0.67
0.5 0.67
0.25 1
cells/well seeded
4 Gy
256
2
256 0.00
128 0.00
64 0.00
32 0.08
16 0.25
8 0.33
4 0.42
2 0.83

Figure 1

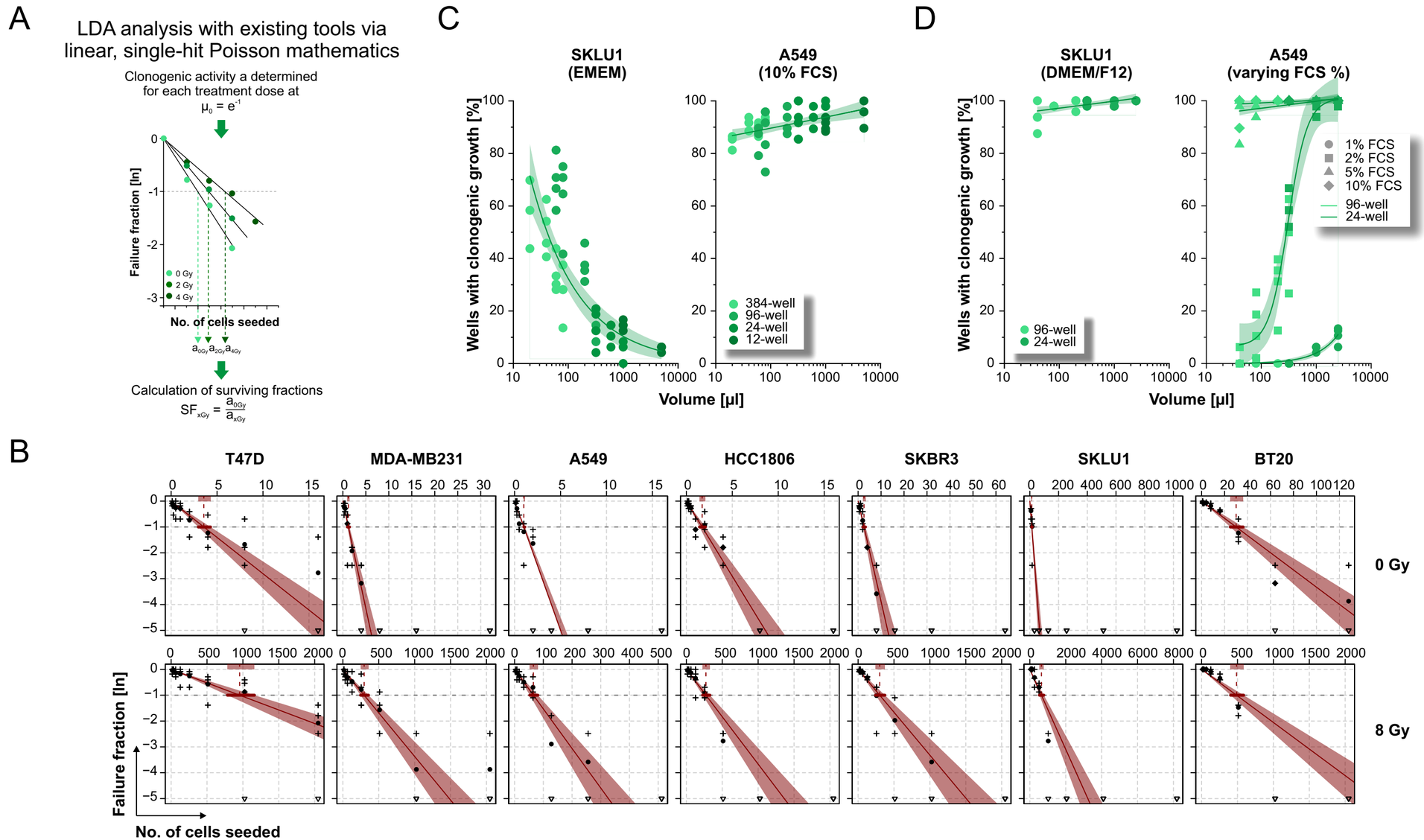

A
LDA analysis with existing tools via linear, single-hit Poisson mathematics
Clonogenic activity a determined for each treatment dose at
$\mu_0 = e^{-1}$
Failure fraction [ln]
0 Gy
2 Gy
4 Gy
No. of cells seeded
a_{0Gy} a_{2Gy} a_{4Gy}
Calculation of surviving fractions
$SF_{xGy} = \frac{a_{0Gy}}{a_{xGy}}$
C
SKLU1 (EMEM)
A549 (10% FCS)
Wells with clonogenic growth [%]
Volume [µl]
384-well
96-well
24-well
12-well
D
SKLU1 (DMEM/F12)
A549 (varying FCS %)
96-well
24-well
1% FCS
2% FCS
5% FCS
10% FCS
B
T47D
MDA-MB231
A549
HCC1806
SKBR3
SKLU1
BT20
0 Gy
8 Gy
Failure fraction [ln]
No. of cells seeded

Figure 2

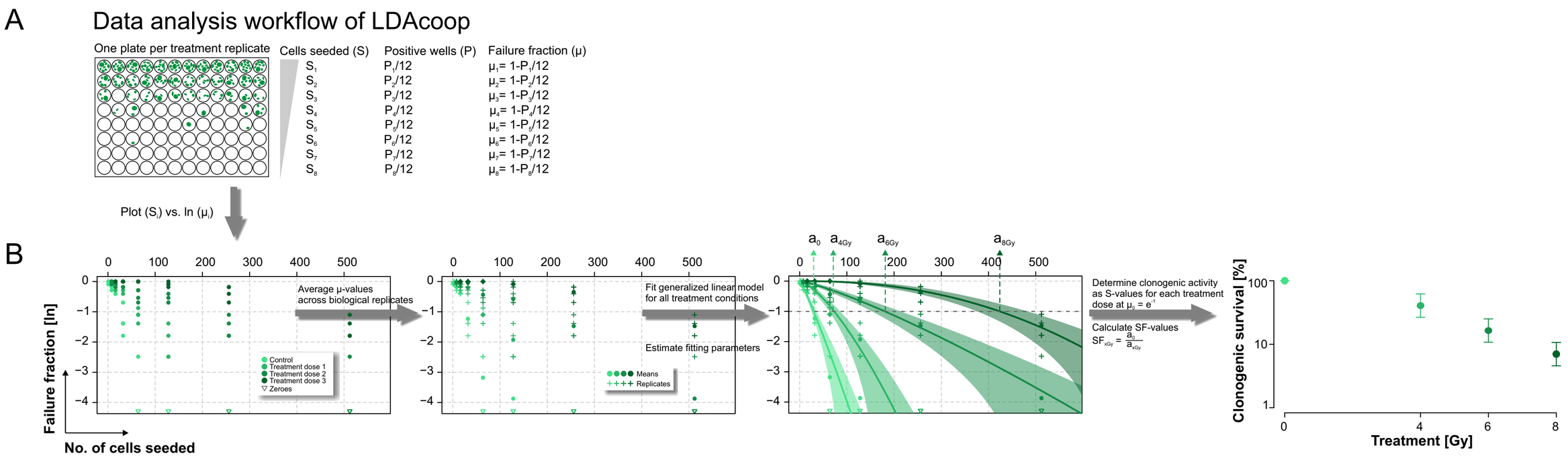
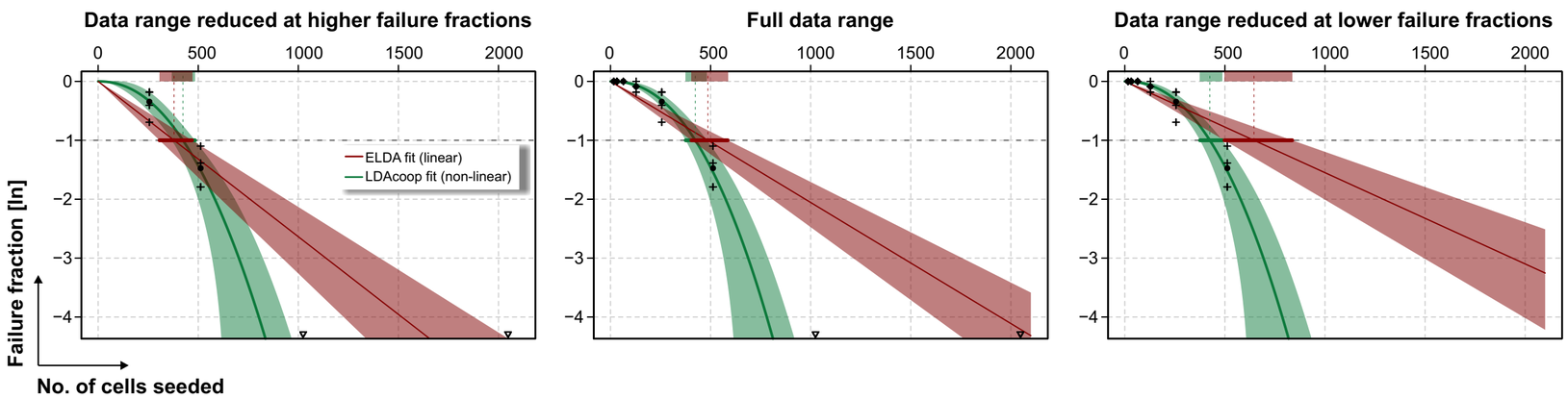

A
Data analysis workflow of LDAcoop
One plate per treatment replicate
Cells seeded (S)
Positive wells (P)
Failure fraction (μ)
S1 P1/12 μ1= 1-P1/12
S2 P2/12 μ2= 1-P2/12
S3 P3/12 μ3= 1-P3/12
S4 P4/12 μ4= 1-P4/12
S5 P5/12 μ5= 1-P5/12
S6 P6/12 μ6= 1-P6/12
S7 P7/12 μ7= 1-P7/12
S8 P8/12 μ8= 1-P8/12
Plot (Si) vs. ln (μi)
B
Failure fraction [ln]
No. of cells seeded
Control
Treatment dose 1
Treatment dose 2
Treatment dose 3
Zeroes
Average μ-values across biological replicates
Means
Replicates
Fit generalized linear model for all treatment conditions
Estimate fitting parameters
a0
a4Gy
a6Gy
a8Gy
Determine clonogenic activity as S-values for each treatment dose at μ0 = e-1
Calculate SF-values
SFxGy = a0 / axGy
Clonogenic survival [%]
Treatment [Gy]

Figure 3

A


Robustness of fit
Data range reduced at higher failure fractions
Full data range
Data range reduced at lower failure fractions
0
500
1000
1500
2000
−1
−2
−3
−4
ELDA fit (linear)
LDAcoop fit (non-linear)
Failure fraction [ln]
No. of cells seeded

B

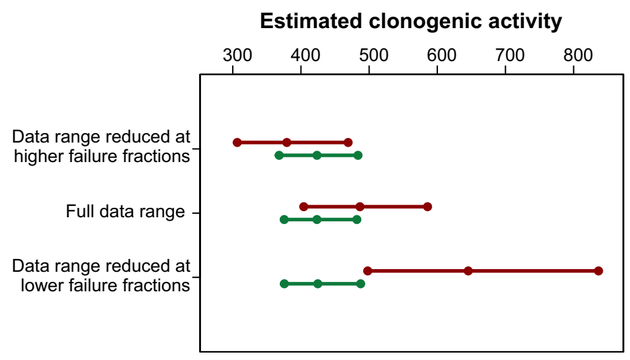

Estimated clonogenic activity
300
400
500
600
700
800
Data range reduced at higher failure fractions
Full data range
Data range reduced at lower failure fractions

Figure 4

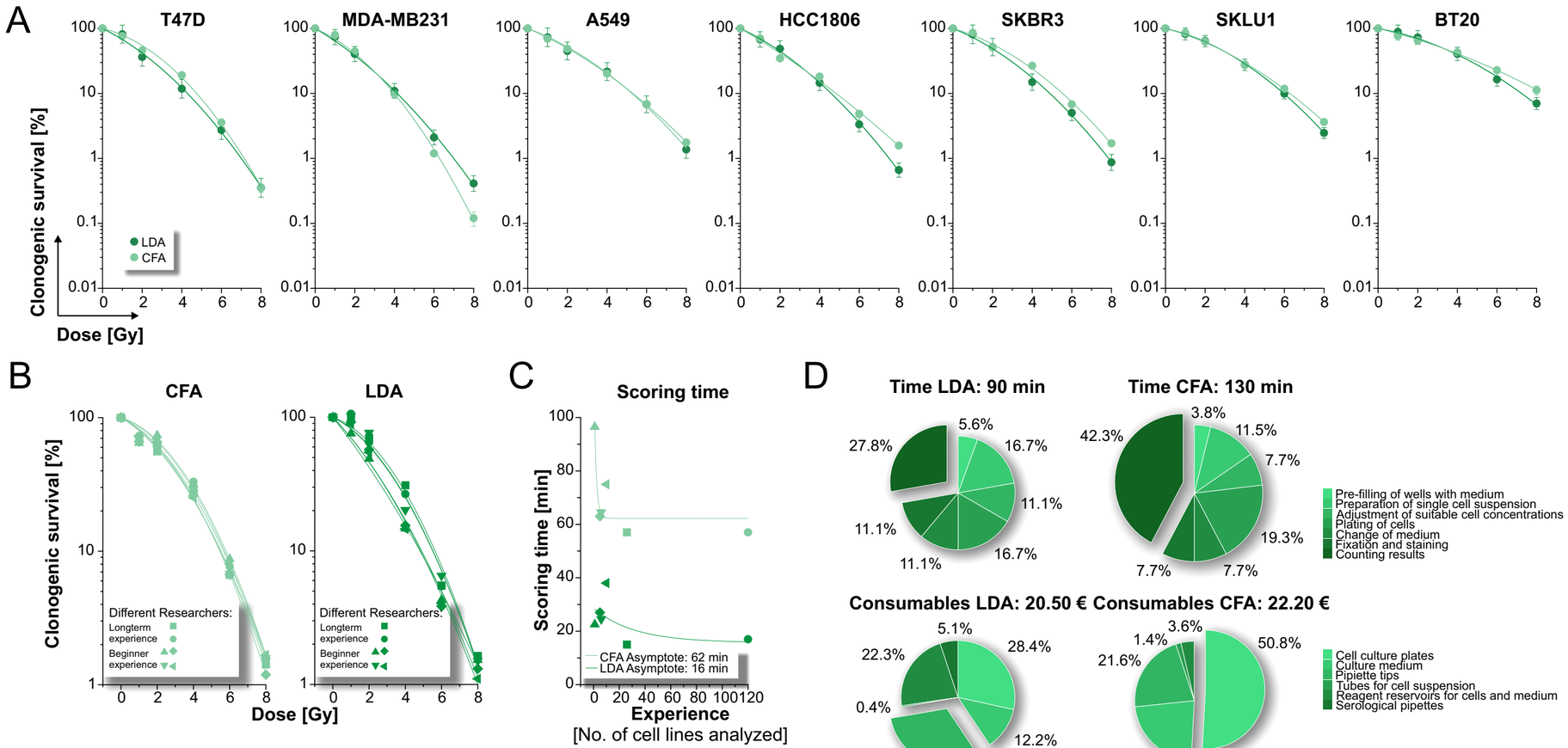

A
T47D
MDA-MB231
A549
HCC1806
SKBR3
SKLU1
BT20
Clonogenic survival [%]
Dose [Gy]
LDA
CFA
B
CFA
LDA
Clonogenic survival [%]
Different Researchers:
Longterm experience
Beginner experience
Dose [Gy]
C
Scoring time
Scoring time [min]
CFA Asymptote: 62 min
LDA Asymptote: 16 min
Experience
[No. of cell lines analyzed]
D
Time LDA: 90 min
Time CFA: 130 min
27.8%
5.6%
16.7%
11.1%
11.1%
11.1%
16.7%
42.3%
3.8%
11.5%
7.7%
19.3%
7.7%
7.7%
Pre-filling of wells with medium
Preparation of single cell suspension
Adjustment of suitable cell concentrations
Plating of cells
Change of medium
Fixation and staining
Counting results
Consumables LDA: 20.50 €
Consumables CFA: 22.20 €
22.3%
5.1%
28.4%
0.4%
12.2%
31.6%
21.6%
1.4%
3.6%
50.8%
22.5%
Cell culture plates
Culture medium
Pipette tips
Tubes for cell suspension
Reagent reservoirs for cells and medium
Serological pipettes

Figure 5

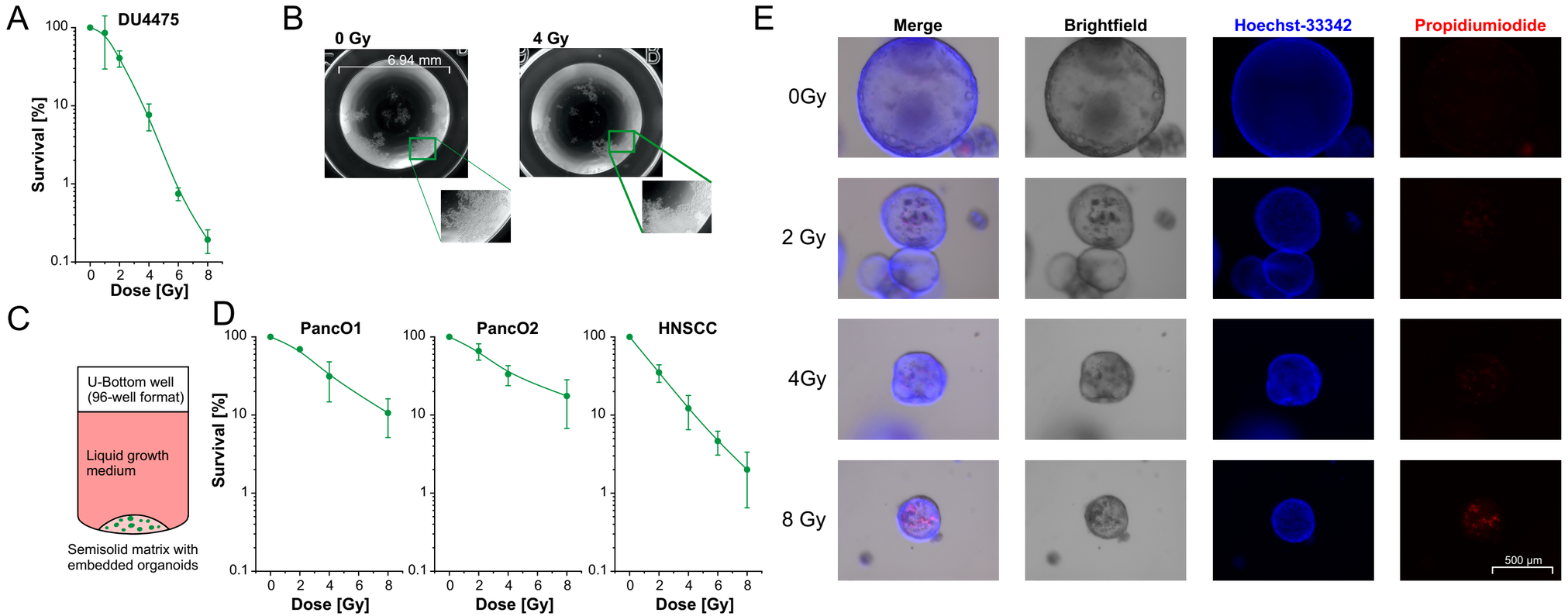

A
DU4475
Survival [%]
Dose [Gy]
B
0 Gy
4 Gy
6.94 mm
C
U-Bottom well (96-well format)
Liquid growth medium
Semisolid matrix with embedded organoids
D
PancO1
PancO2
HNSCC
Survival [%]
Dose [Gy]
E
Merge
Brightfield
Hoechst-33342
Propidiumiodide
0Gy
2 Gy
4Gy
8 Gy
500 µm

Figure 6

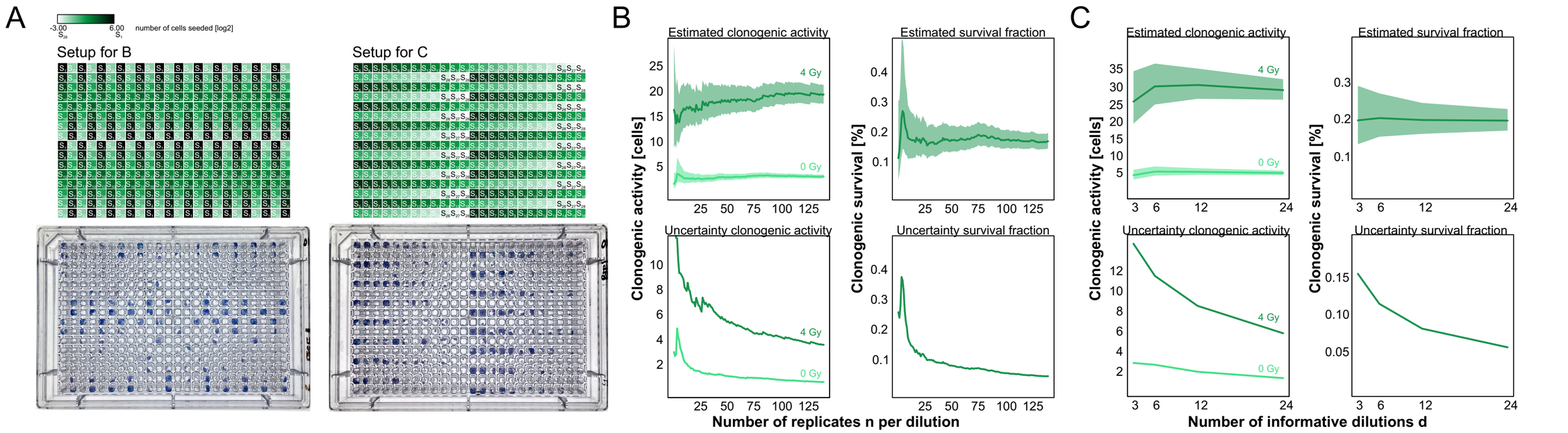

A
-3.00
6.00
number of cells seeded [log2]
Setup for B
Setup for C
B
Estimated clonogenic activity
Estimated survival fraction
4 Gy
0 Gy
Clonogenic activity [cells]
Clonogenic survival [%]
Uncertainty clonogenic activity
Uncertainty survival fraction
Number of replicates n per dilution
C
Estimated clonogenic activity
Estimated survival fraction
Uncertainty clonogenic activity
Uncertainty survival fraction
Number of informative dilutions d

Figure 7

Supplemental Tables, Figures, and References

Supplemental Table 1: Overview of cell culture media used for adherent and suspension cells. "P/S" corresponds to 100 U/mL penicillin and 0.1 mg/mL streptomycin.

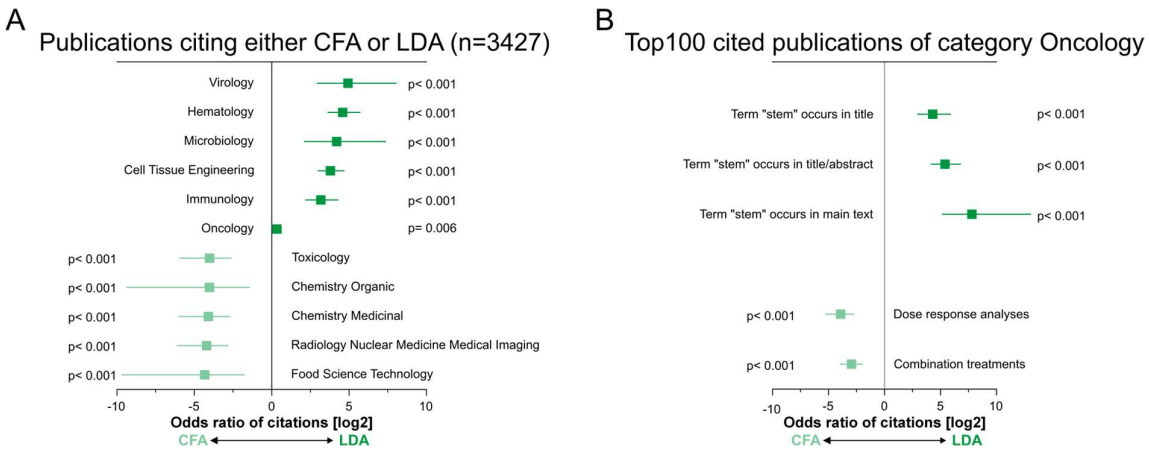
Cell line	Supplier	Culture medium	FCS [%]	CO_2 [%]
T47D	CLS	RPMI 1640 (GlutaMAX™ + 35 mM HEPES) + P/S	10	5
SKBR3	CLS	DMEM (high glucose, GlutaMAX™, pyruvate) + P/S	10	7.5
BT20	CLS	DMEM/F-12 (1 + 1, GlutaMAX™) + P/S	10	7.5
MDA-MB231	ATCC	DMEM (high glucose, GlutaMAX™, pyruvate) + P/S	10	7.5
HCC1806	ATCC	RPMI 1640 (GlutaMAX™ + 35 mM HEPES) + P/S	10	5
DU4475	DSMZ	RPMI 1640 (GlutaMAX™ + 35 mM HEPES) + P/S	20	5
A549	DSMZ	DMEM (high glucose, GlutaMAX™) + P/S	10	7.5
SKLU1	CLS	EMEM basic medium + P/S	10	5

Supplemental Table 2: Formulations of PDAC organoid media according to [1].

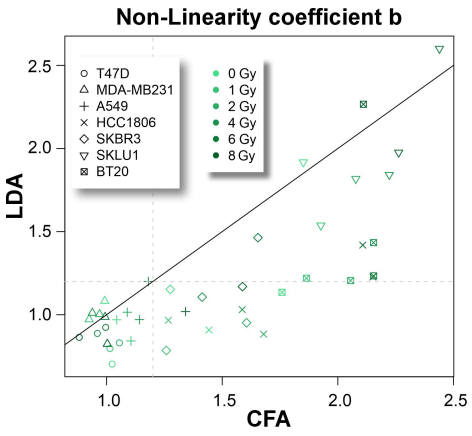
Base Medium	Supplier	
Advanced DMEM/F-12	Thermo Fisher 12634010	
HEPES	Sigma H 0887	1%
GlutaMAX	Thermo Fisher 35050061	1%
Primocin	Invivo Gen ant-pm-05	100 µg/ml
Reagent	Supplier	Concentration
Base medium		35.5%
WNT3a conditioned medium		50%
R-spondin conditioned medium		10%
Nicotinamide	Sigma-Aldrich N 0636	10 mM
B27 Supplement	Thermo Fisher 17504044	1x
Human FGF10	PeproTech 100-26	100 ng/ml
Human EGF	PeproTech AF-100-15	50 ng/ml
Murine Noggin	PEPRO 120-10C-20	100 ng/ml
A83-01	Sigma-Aldrich SML0788	0.5 µM
Prostaglandin E2	Tocris TOC 2296/10	1 µM
Human Gastrin	Sigma-Aldrich G9020	10 nM
N-acetyl-L-cysteine	Sigma-Aldrich A9165	1.25 mM

Supplemental Table 3: Formulation of HNSCC organoid media according to [2, 3].

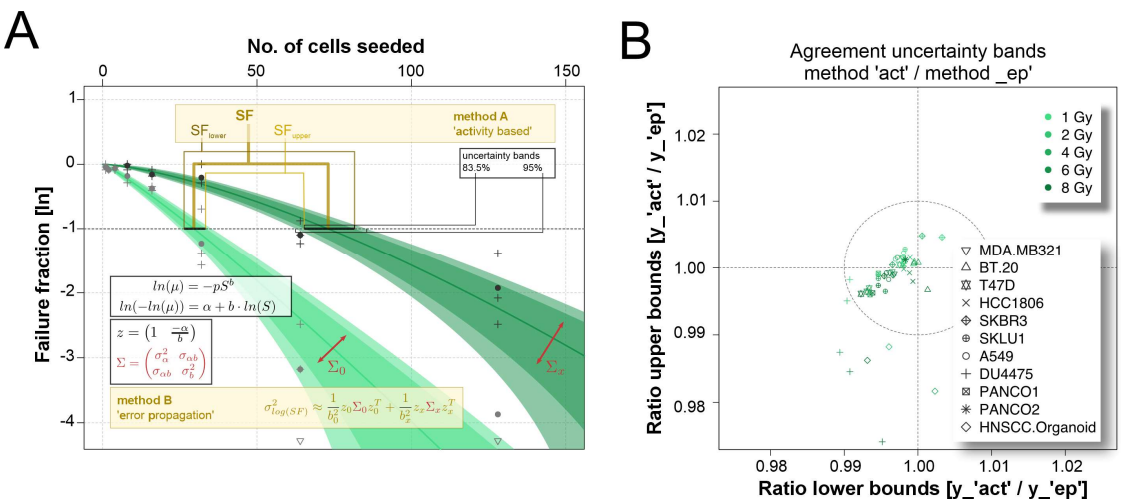
Base Medium	Supplier	
Keratinocyte SFM	Thermo Fisher Scientific 17005042	
Reagent	Supplier	Concentration
Bovine Pituitary Extract	Thermo Fisher Scientific 17005042	50 µg/ml
Human EGF	Thermo Fisher Scientific 17005042	1 ng/ml
$CaCl_2$	Sigma-Aldrich C5080	0.6 mM
Primocin	Invivo Gen ant-pm-05	100 µg/ml



Supplemental Figure 1: The use of CFA and LDA is highly unevenly distributed across different research disciplines in life sciences. A) Publications citing the two most-cited research papers describing either the CFA ("Clonogenic assay of cells *in vitro*" by Franken et al. [4], n = 2,329 citations) or the LDA ("ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays" by Hu and Smyth [5] n = 1,098 citations) were assigned to different research disciplines using the Web of Science platform (Clarivate). The $\log_2$ odds ratios ($\log_2$ [ratio of citations in research discipline X of Hu and Smyth to citations of Franken et al. relative to the ratio of citations in other fields citing one of those]) of different fields are shown. Statistical differences in citation behavior were assessed by Fisher's exact test, and *p*-values are indicated. **B)** Use of CFA and LDA in cancer research. All citing papers of Franken et al. [4] and Hu and Smyth [5] in the Web of Science category "Oncology" as shown in A) were sorted in descending order by their number of citations. The top 100 primary research papers with *in vitro* use of CFA (n= 100) or LDA (n= 100) were analyzed for the occurrence of the term "stem" (upper panel) and the detailed type of analysis with regard to dose-response or combination treatment analysis (lower panel). For details on data acquisition and scoring, see Materials and Methods section. Statistical differences between groups were analyzed by Fisher's exact test, and *p*-values are shown.



Supplemental Figure 2: Comparison of the non-linearity coefficients b of clonogenic growth behavior as determined in the CFA and LDA format. Non-linearity coefficients of all cell lines were determined at different treatment conditions in the CFA and LDA format and calculated via CFAcoop and LDAcoop, respectively. Both CFA and LDA data of all cell lines were generated in three to four independent biological replicates of which mean b-value pairs (b_{CFA} | b_{LDA}) are displayed for each radiation dose.



Supplemental Figure 3: Methods of uncertainty estimation implemented in LDAcoop A) Uncertainties of surviving fractions as estimated based on activity (method A) as described in the Materials and Methods section or via error propagation (method B). Both methods are available in LDAcoop. **B)** Comparison of uncertainty bands obtained via method A and method B demonstrates high agreement of both methods. The ratio [uncertainties obtained by method A / uncertainties obtained by method B] of both lower(x-axis) and upper bound (y-axis) are depicted.

References

1. Mahajan UM, Li Q, Alnatsha A, Maas J, Orth M, Maier SH, et al. Tumor-Specific Delivery of 5-Fluorouracil-Incorporated Epidermal Growth Factor Receptor-Targeted Aptamers as an Efficient Treatment in Pancreatic Ductal Adenocarcinoma Models. Gastroenterology. 2021;161(3):996-1010 e1.
2. Weber P, Kunstner A, Hess J, Unger K, Marschner S, Idel C, et al. Therapy-Related Transcriptional Subtypes in Matched Primary and Recurrent Head and Neck Cancer. Clin Cancer Res. 2022;28(5):1038-52.
3. Kasagi Y, Chandramouleeswaran PM, Whelan KA, Tanaka K, Giroux V, Sharma M, et al. The Esophageal Organoid System Reveals Functional Interplay Between Notch and Cytokines in Reactive Epithelial Changes. Cell Mol Gastroenterol Hepatol. 2018;5(3):333-52.
4. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. 2006;1(5):2315-9.
5. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009;347(1-2):70-8.