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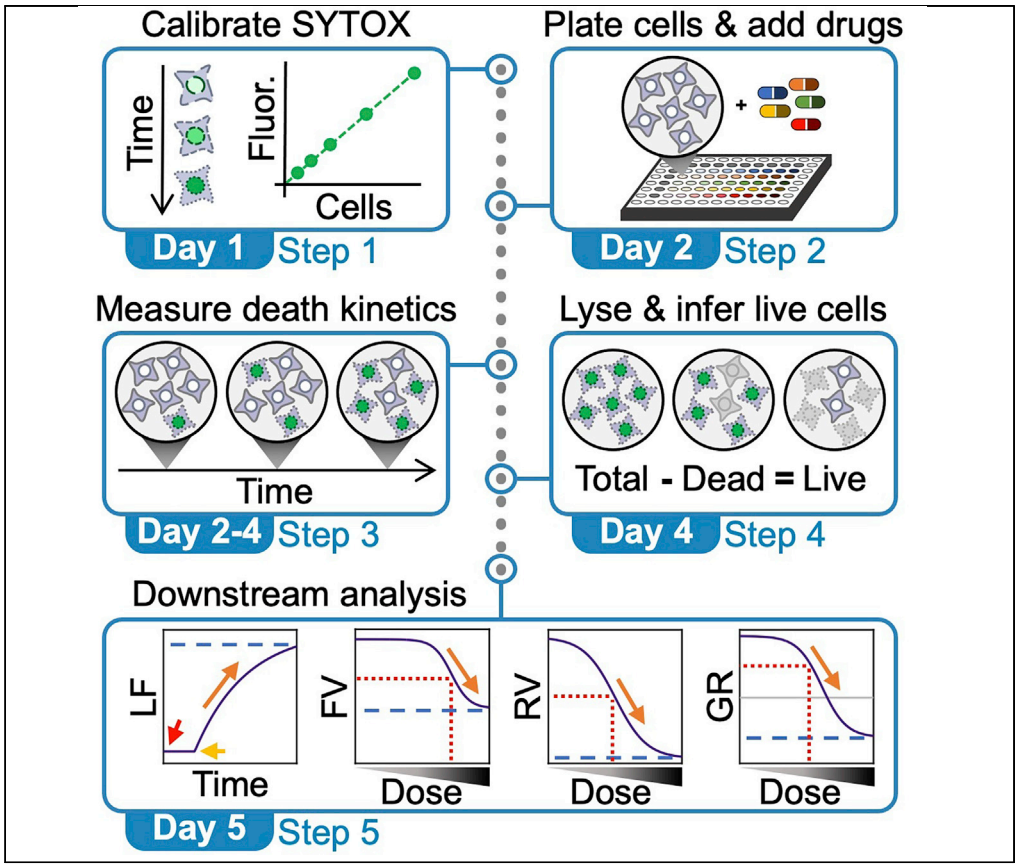
FLICK: An optimized plate reader-based assay to infer cell death kinetics



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Ryan Richards, Megan E. Honeywell, Michael J. Lee

ryanarichards87@gmail.com (R.R.)
michael.lee@umassmed.edu (M.J.L.)

HIGHLIGHTS

FLICK generates a death-specific measure of drug response not a correlate of viability

FLICK is high-throughput and easy to use like common plate reader-based assays

FLICK can be used to generate any commonly used drug response metric

FLICK can be used to infer cell death kinetics

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FLICK: An optimized plate reader-based assay to infer cell death kinetics

Ryan Richards,[1,2,3,*] Megan E. Honeywell,[1] and Michael J. Lee[1,4,*]

[1]Program in Systems Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA

[2]Present address: AstraZeneca, 35 Gatehouse Drive, Waltham, MA 02451, USA

[3]Technical contact

[4]Lead contact

*Correspondence: ryanarichards87@gmail.com (R.R.), michael.lee@umassmed.edu (M.J.L.)
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SUMMARY

Evaluating drug sensitivity is improved by directly quantifying death kinetics, rather than correlates of viability, such as metabolic activity. This is challenging, requiring time-lapse microscopy and genetically encoded labels to distinguish live and dead cells. Here, we describe fluorescence-based and lysis-dependent inference of cell death kinetics (FLICK). This method requires only a standard fluorescence plate reader, retaining the high-throughput nature and broad accessibility of common viability assays. However, FLICK specifically quantifies death, including an accurate inference of death kinetics.

For complete details on the use and execution of this protocol, please refer to Richards et al. (2020).

BEFORE YOU BEGIN

Calibrate the use of SYTOX for cells of interest

Timing: 1 day

1. In two 96-well, optical bottom, black-walled plates, add to the first column of both plates 40,000 cells in 180 μL of cell culture media (16 wells total). This first well will be used to generate a 2-fold cell dilution across the plate. The final concentration for this first well will be 20,000 cells/well (i.e., 20,000 cells per 90 μL).

   Note: This protocol has been written for use with a Tecan Spark multimode plate reader, with fluorescence readings taken from the bottom of the plate. Optical bottom plates are not necessary if the plate reader used acquires fluorescence emission from the top of the wells. However, even in this case, optical bottom plates may still be helpful for visualizing cell lysis (see Before you begin, Step 8). Additionally, this protocol has been optimized for U2-OS cells. Testing an altered range of cell densities may be optimal for other types of cells.

   ⚠ CRITICAL: Media containing phenol red has an insignificant effect on the SYTOX fluorescence signal or signal linearity. Other cell line-specific media reagents should be checked for auto-fluorescence at 504/523 nm excitation and emission wavelengths.

2. Add 90 μL of media to all remaining wells in both plates (176 wells total).
3. Serially dilute cells 1:2 from the first column of the plates into the adjacent columns by removing 90 μL and adding it to the adjacent column to the right. Continue the dilution until reaching the

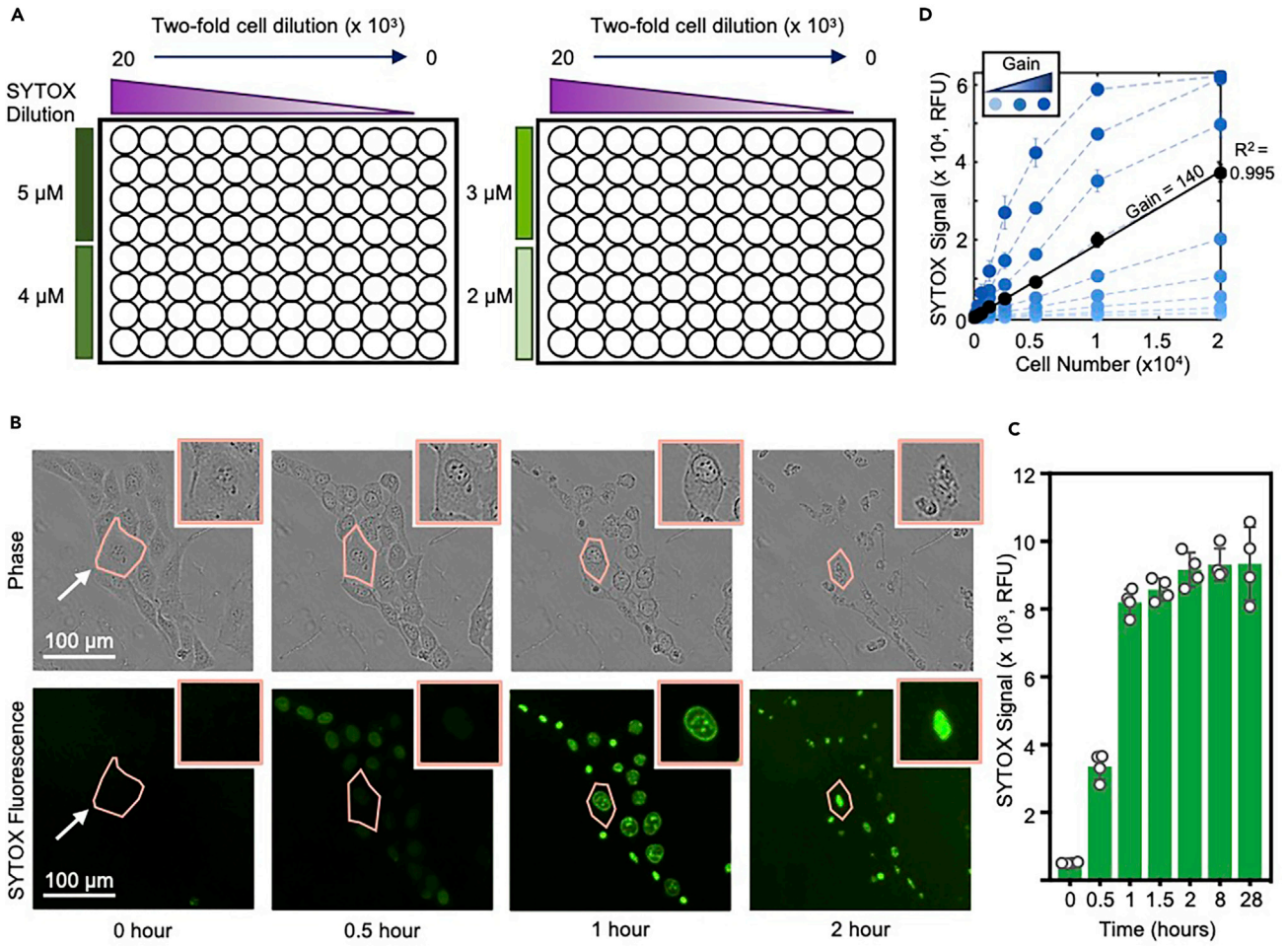

Figure 1. Optimizing SYTOX dyes for measuring cell death in a fluorescence plate reader

(A) Plating design for establishing a linear range and acquisition settings for SYTOX fluorescence. SYTOX dilution represented using green shades. Cell dilution represented using blue shades.

(B) Visual inspection of cell permeabilization by Triton. Phase and SYTOX fluorescence images shown for cells exposed to 0.1% Triton-X. Images collected using a 10× objective. Scale bar applies to all images, which are at the same magnification. Cell boundary for the highlighted cell was added for illustrative purposes and is not part of the analysis. Inset image of the highlighted cell is a cropped 2× version of the same image.

(C) Optimal permeabilization times and degree of signal stability after cell lysis was determined by measuring SYTOX fluorescence at varied times after cell permeabilization. Data are mean fluorescence in the well ±SD, with data from 4 individual replicate wells overlayed.

(D) Establishing acquisition settings for SYTOX fluorescence. Linearity of SYTOX fluorescence evaluated at varied fluorescence gain settings (90–170). SYTOX signal at a gain of 140 highlighted in black. Data are mean ±SD of replicates following a 2 h Triton permeabilization with 5 µM SYTOX green, using the plating design shown in (A).

11th column. The last column should not contain any cells on either plate and is used for determining background fluorescence signal.

a. Discard 90 µL from the 11th column wells.

Note: At this point all wells should have 90 µL of media. The first column should have 20,000 cells; the 11th column should have approximately 20 cells; the last column should contain only media (Figure 1A).

4. Incubate plates at 37°C and 5% CO_2 for >4 h until the cells are adhered. Incubation time may be longer or shorter depending on cell line of interest.
5. Prepare SYTOX reagent at 10× final concentration.

a. Prepare 10× SYTOX solutions in media at the following concentrations: 50, 40, 30, and 20 μM.
   i. Add 10, 8, 6, or 4 μL of 5 mM SYTOX green to 1 mL of culturing media to create the 50, 40, 30, and 20 μM 10× stock solutions, respectively.

Note: SYTOX is used in this protocol to label dead cells. SYTOX fluorescence increases upon binding to DNA, and fluorescence depends on loss of plasma membrane integrity. This general mechanism is beneficial, as SYTOX can be used to evaluate the degree of cell death independently from the mechanism of cell death. The FLICK method we describe for inferring death kinetics can be used with any dye that labels dead cells independent of mechanism. Other dyes that label dead cells in a death mechanism-specific manner (e.g., apoptotic specific) may be valuable for quantifying the kinetics of activation for specific death mechanisms.

6. Prepare 10× Triton-X permeabilization buffer.
   a. Make a 10× Triton-X solution in cell culture grade PBS buffer by adding 50 μL Triton-X to 5 mL PBS (1.0% solution). Mix the solution by inverting the tube. Avoid shaking.

Note: To help dissolve Triton-X, the solution can be put in a 37°C water bath for 5 min. The final concentration of Triton-X when added to cells can be between 0.05%–0.15%. The 10× solution should adjusted accordingly if using a final concentration other than 0.1%.

7. Add 10× SYTOX solutions (Figure 1A).
   a. Each 10× SYTOX solution will be added to 4 rows of cells creating quadruplicate measurements for each dilution (Figure 1A).
      i. To the first 4 rows on plate 1, add 10 μL of 50 μM 10× SYTOX solution from step 5 for a final concentration of 5 μM.
      ii. To the last 4 rows on plate 1, add 10 μL of 40 μM 10× SYTOX solution from step 5 for a final concentration of 4 μM.
      iii. To the first 4 rows on plate 2, add 10 μL of 30 μM 10× SYTOX solution from step 5 for a final concentration of 3 μM.
      iv. To the last 4 rows on plate 2, add 10 μL of 20 μM 10× SYTOX solution from step 5 for a final concentration of 2 μM.

Note: These data will be used to optimize the concentration of SYTOX and the fluorescence acquisition settings on the plate reader. The technical replicates are not intended to capture other sources of biological or experimental variation. See Optimizing Experimental Design, step 14 for details on optimizing plating designs.

8. Permeabilize cells (Figures 1B and 1C).
   a. Add 10 μL of 1% Triton-X solution prepared in step 6 to all wells for a final concentration of 0.1%.
   b. Place plates in incubator at 37°C and 5% CO_2 for approximately 1.5–2 h to allow for cell permeabilization.

⚠ CRITICAL: The timing for permeabilization will vary depending on cell line and Triton-X concentration. It may be necessary to incubate longer to ensure complete cell lysis. Check for cell permeabilization under a microscope (Figure 1B). Following lysis, cell morphology should be significantly altered. No discernable intact cells should remain in the well, with only cell debris remaining (see Figure 1B, 2 h). A key observation is the change in nuclear morphology and lack of clear nuclear boundary (see Figure 1B, change between 1 and 2 h). Even following dramatic changes to nuclear morphology, total well fluorescence is largely unaffected (Figure 1C). If fluorescence and/or phase microscopy is not available, permeabilization time can be optimized by reading the plate over time, to determine when the signal hits a stable plateau.

Table 1. Example SYTOX calibration settings (all using 5μM SYTOX green)

Cell	Permeabilization time (h)	Gain
A549	2	95
BT-20	3	130
HeLA	2	95
MCF10A	2	130
MCF7	2	95
MDA-MB-231	4	120
MDA-MB-468	3	130
PC9	3	120
Primary Human Fibroblast	3	140
T47D	4	130
U2-OS	2	130

9. Set up the plate reader for quantification of SYTOX fluorescence.
   a. The excitation and emission wavelengths should be set to 504/523 nm with a bandwidth of 5 nm, if possible.
   b. Flash number should be set at 5.

   Note: The settings above are specified for the Tecan Spark. Settings will vary based on the plate reader. Some models may allow control of fluorescence integration time rather than flash number. Integration time should be kept short to minimize photobleaching.

   ⚠ CRITICAL: Once cells are lysed, the SYTOX signal is stable (Figure 1C). Avoid shaking or agitating the plate at this step, which can result in dislodging/aggregation of the cells, leading to inaccurate readings in some plate readers (Grootjans et al., 2016).

10. Collect SYTOX fluorescence using range of gain settings (Figure 1D).
    a. For each of the four SYTOX concentrations, collect fluorescence data using a spectrum of gain values. Gain settings should include levels that produce low signals at all cell numbers, through gain settings that produce saturated fluorescence signals in wells containing high cell numbers. Saturated signals are reported as "invalid" (INV) or "NaN" in most plate readers, and refer to signals that are above the maximum detectable limit (65,000 fluorescence units for the Tecan Spark). Refer to the plate reader manual to determine the maximum fluorescence signal that can be detected.
11. Data analysis to determine optimal acquisition settings.
    a. Remove the background fluorescence from each measurement by calculating the mean signal from wells that do not contain cells; subtract this mean value from the signal for all wells.
    b. For each SYTOX concentration and gain condition, compare the background subtracted fluorescence signal to the cell number (Figure 1D).
    c. Determine the correlation coefficient (R^2) for each condition using linear regression.
       i. In MATLAB, the built-in function "corrcoef" can be used to compute the Pearson correlation coefficient (R).
       ii. In Excel, correlation coefficient can be calculated using the function "CORREL."
    d. Select the SYTOX concentration and gain setting that gives the best combination of linearity and dynamic range.
       i. The maximum signal (wells that contain 20,000 cells) should be less than 80%–90% of the detection limit (65,000 relative fluorescence units on the Tecan Spark).
       ii. In most titration experiments a range of gain values produce large dynamic range and $R^2 >$ 0.99 (Figure 1D, gain values between 120–140 are all acceptable). See Table 1 for example settings used for other cell lines.

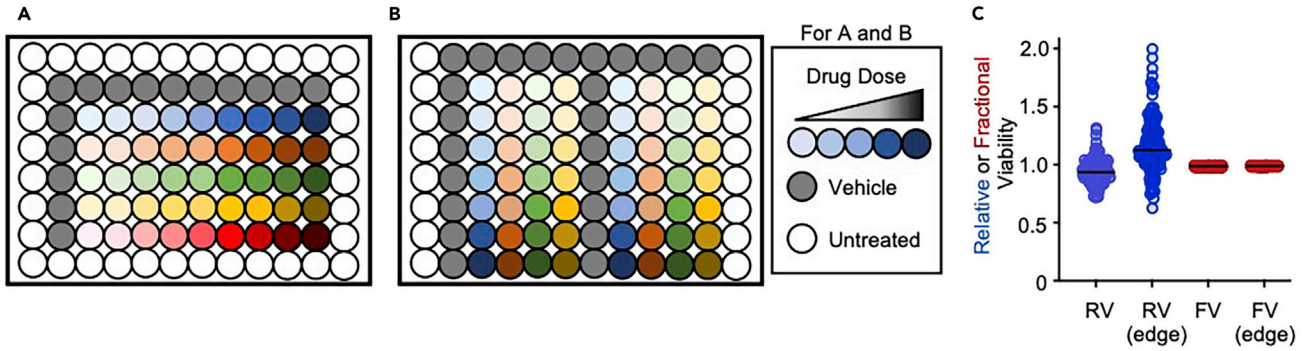

Figure 2. Optimal plating designs for analysis of drug sensitivity using common pharmacometrics

(A and B) Schematics of 96-well plating layouts containing drugs at varied doses (graded colors), vehicle controls (gray), and untreated wells (white). (A) Edge wells are often omitted due to different growth characteristics. Vehicle controls arrayed systematically across the plate can assist with identifying/normalizing plating biases if needed. This standard design has no technical replicates and biological replicates located on separate plates. (B) Partial use of edge wells, with high doses of drug on one edge, and vehicle controls across top edge and two columns of the plate. Using this design, normalization to vehicle controls should be performed using the plate mean across all vehicle wells to minimize edge effects.

(C) Variable influence of plating design, dependent on choice of downstream analysis metrics. Data shown for relative viability (RV or RV edge; defined as live cells in treated wells compared to control) or Fractional Viability (FV or FV edge; defined as live cells in each well compared to total of live and dead cells in the same well). Data are for DMSO treated wells using a plating design, similar to (A). For RV, DMSO treated wells were normalized relative to the plate average for all DMSO treated wells. For RV edge, DMSO treated wells were normalized relative to the average of all untreated wells, which were located on the plate edge. FV refers to the fraction of live cells in DMSO treated wells. FV edge refers to the fraction of live cells in edge wells. Use of edge wells in normalization increases variability of RV but not FV metrics. Data are shown for 150 individual wells with median values highlighted with a black line.

Optimize experimental design

Timing: 1 day

12. Option 1 – Set up a T0 control plate to determine live and dead cell numbers at assay start.
    a. When designing an experiment to calculate lethal fraction over time, a T0 control plate should be used to estimate the initial lethal fraction, the proportion of cells that are alive versus dead, at the start of the assay. This plate is seeded at the same time as experimental plates with the same number of cells, but will be lysed at the start of the experiment. An entire 96 well plate should be used for this calculation (see Quantification and statistical analysis step 2).
13. Option 2 – Estimate the live and dead cells at assay start from previous experiments.
    a. Fluorescence readings from a previous experiment can be used as an approximation to determine the initial cell counts if the same number of cells were seeded in both experiments.

⚠ CRITICAL: Do not lyse a subset of wells on an experimental plate as the T0 control. The lysis buffer can permeate to other wells across the plate over time, causing lysis.

14. Consider the optimal plating layout (Figures 2A and 2B).
    a. Evaporation and slow growth often occur on the outer edges of the plate. We have observed that drug-induced lethal fraction is not generally altered by growth rate. Additionally, because lethal fraction is internally controlled (i.e., does not require comparison between two wells), the outer edges can be used if downstream analyses focus exclusively on lethal fraction. However, one should expect lower total cell counts in these wells. Relative viability, which requires comparison between separate wells on a plate should not be calculated using the edge wells (Figure 2C).
    b. Other sources of variability in this assay could include inconsistencies in cell plating between plates or on separate days. Additionally, variations in Triton permeabilization efficiency may

exist for plates that are lysed at different times or from different permeabilization buffers. The plating design should avoid confounding theses sources of variation with treatment groups.

Note: The T0 control plate can be used to correct for the growth variability if necessary. This should only be done if users have validated that a systematic plating location bias reproducibly occurs on a given plate reader.

15. Delivering compound and SYTOX reagent.
    c. When possible, 10× compound stocks should be made in media containing 10× SYTOX. This allows the operator to dispense both reagents into the well in a single pipette stroke. The cell plating volume should be adjusted so a final volume of 100 μL in the well is achieved, after adding SYTOX and compound.

Note: 100 μL final volume is used in order to limit the amount of reagents needed, and because U2-OS cells grow at expected rates when plated at these densities in this volume. Plating volumes can be adjusted if needed, based on the growth characteristics of the cells in the study.

⚠ CRITICAL: At high concentrations of drug, the percentage of DMSO may be above the tolerance of cells. It is important to have vehicle controls on the plate to determine the contribution of death from DMSO.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
SYTOX green	Thermo Fisher Scientific	Cat# S7020
Triton	Fisher BioReagents	Cat# BP151-500
DMEM	Corning	Cat# 10017CV
SGI-1027	ApexBio	B1622
Camptothecin	Sigma	C9911
Deposited data		
fitGrowth function	Richards et al., 2020	https://github.com/MJLee-Lab/fitGrowth
fitLED function	Richards et al., 2020	https://github.com/MJLee-Lab/fitLED
fit_via function	Richards et al., 2020	https://github.com/MJLee-Lab/fit_via
Experimental models: cell lines		
U2-OS	ATCC	HTB-96
Software and algorithms		
MATLAB	MathWorks	R2019b
fitGrowth function	Richards et al., 2020	https://github.com/MJLee-Lab/fitGrowth
fitLED function	Richards et al., 2020	https://github.com/MJLee-Lab/fitLED
fit_via function	Richards et al., 2020	https://github.com/MJLee-Lab/fit_via
PRISM	GraphPad	Version 9.0.0
Excel	Microsoft	Excel for Mac version 16.42
Other		
Microplate fluorescence reader	Tecan	Spark
Optical bottom black-walled 96 well plates	Greiner Bio-One	655090

MATERIALS AND EQUIPMENT

Alternatives: This protocol uses a Tecan Spark Multimode plate reader. Essentially any other plate reader should also be suitable. The minimal requirement is only the ability to quantify a

single channel of fluorescence associated with labeling of dead cells. Labeling of dead cells in this protocol is performed using the SYTOX green dye. SYTOX dyes in other colors are also suitable, assuming these can be read in the chosen plate reader. SYTOX dyes are used in this protocol as their fluorescence is increased when bound to DNA in dead cells, and these dyes are not toxic to live cells, enabling kinetic analysis. Other dyes with these features should also be usable with appropriate titration.

STEP-BY-STEP METHOD DETAILS

Plating cells

Timing: 20 min

Following experimental design, cells should be grown and collected to be plated into 96 well, optical bottom, black-walled plates. The number of plates needed is dictated by the number of conditions to be tested; however, an additional T0 control plate must also be plated. Care should be taken in accurately counting and seeding cells. This will reduce noise in the fluorescence measurements and provide consistent data between replicates. Two technical replicates are performed on the same plate to account for plating bias. Additionally, biological replicates should also be performed on separate plates or experiments performed on subsequent days.

1. Cell line of interest should be cultured according to established protocols.

   Note: This protocol is suitable for cells with any growth morphologies or characteristics. Very small cells or cells that grow with uneven density (clumpy or in colonies) will generate noisier data, particularly at low cell densities. This may compromise the low end sensitivity of the assay.

2. Count cells using a hemocytometer or automatic cell counter.
3. Dilute cells to the appropriate concentration in cell culture media that allows for the proper seeding density. Cells should be seeded at a starting density at which untreated cells do not become confluent during the assay, taking into account the doubling time, well size, and length of the experiment. Following counting, cells should be resuspended at a concentration that allows for plating at 90 μL/well.
4. Resuspend cells by gently pipetting up and down, then add 90 μL of cell suspension to plates.
   a. See step 14 in Before you begin and Figure 2 for optimal experimental designs, including number of technical and/or biological replicates per experiment.
5. Place in incubator at 37°C and 5% CO_2 to adhere overnight (4–18 h).

   Pause point: The assay can begin immediately after the cells have adhered to the plate. Most commonly, drugs are added the following morning after plating, but the assay could also start anytime within the following 2–3 days while the wells are not confluent and the cells are growing normally. Whatever decision is made should be consistent across all plates in a given experiment.

Adding SYTOX and compound

Timing: 1 h

SYTOX green solutions with and without compound are prepared and added to experimental wells. A 1% Triton-X solution is prepared and added to the T0 control plate to determine initial total cell fluorescence.

6. Prepare a 1% Triton-X permeabilization buffer in sterile PBS.

a. Approximately 1 mL is required to permeabilize an entire 96-well plate. Extra should be made to account for dead volume, particularly if dispensed from a reservoir using a multi-channel pipette.

7. Prepare a 10× SYTOX solution (see Before you begin step 11) by diluting 5 mM SYTOX in enough volume of culture media for your experimental design. The total volume should be enough for all dilutions, experimental wells, and T0 control plate.
8. Dilute the compounds of interest to 10× the final desired concentration in 10× SYTOX solution. This should also be done for a vehicle control. If doing dose-response experiments, it is recommended to create 10× drug dilutions in round-bottom plates by serial dilution.

Note: Round-bottom plates are recommended to reduce the dead volume during pipetting

9. Add 10 μL of the SYTOX+compound (or vehicle) solution to appropriate wells. Add 10 μL of 10× SYTOX only solution to the T0 control plate. The final volume of the well should now be 100 μL with 1× SYTOX and 1× compound (or vehicle).
10. Add 10 μL of 1% Triton-X permeabilization buffer to all wells of the T0 plate and place in the incubator at 37°C and 5% CO_2 for >2 h (or the length of time optimized in Before you begin, step 8).

Fluorescence measurements

Timing: 1–3 days

This step quantifies the raw fluorescence of dead cells at chosen time points. After the final measurement is taken, cells are lysed using Triton-X buffer to quantify the total cell fluorescence at the end of the assay.

11. Set the fluorescence plate reader to the appropriate excitation, emission, and gain settings for the chosen SYTOX concentration.
12. Take an initial reading for the experimental plates. Once the measurement is finished, keep the plate stored in the incubator at 37°C and 5% CO_2 until the next time point.
13. Once the T0 control plate is fully permeabilized (step 10), measure the fluorescence. This measurement of Total Starting Fluorescence will be used later in Quantification and statistical analysis, step 2. Discard the plate when finished.
14. Continue taking measurements at selected time points until the end of the experiment.
    a. Common assay lengths are generally 48–72 h after drug addition, but these depend on the growth rate of cells being tested.
    b. Time points should be ~ 4 h apart, at a roughly constant interval, and selected to capture multiple (2–3) time points within the increasing phase of the response and the plateau phase of the response. Selected time points should be the same across biological replicates.

Note: Both the frequency and the interval of measurements will affect the accuracy of the kinetic fitting (Quantification and statistical analysis, step 3). In particular, the inference of death onset time (D_O) depends on capturing changes in cell death within both the increasing phase and plateau phase of the response. See Troubleshooting for more details. Plates should remain in the incubator between time point measurements unless the plate reader used is equipped with a temperature and CO_2 controlled environmental chamber. Temperature fluctuations caused by frequent removal from the incubator may compromise cell health and/or growth rate.

15. Prior to reading the final time point, prepare fresh 1% Triton-X permeabilization buffer. Prepare enough volume to lyse all experimental wells.

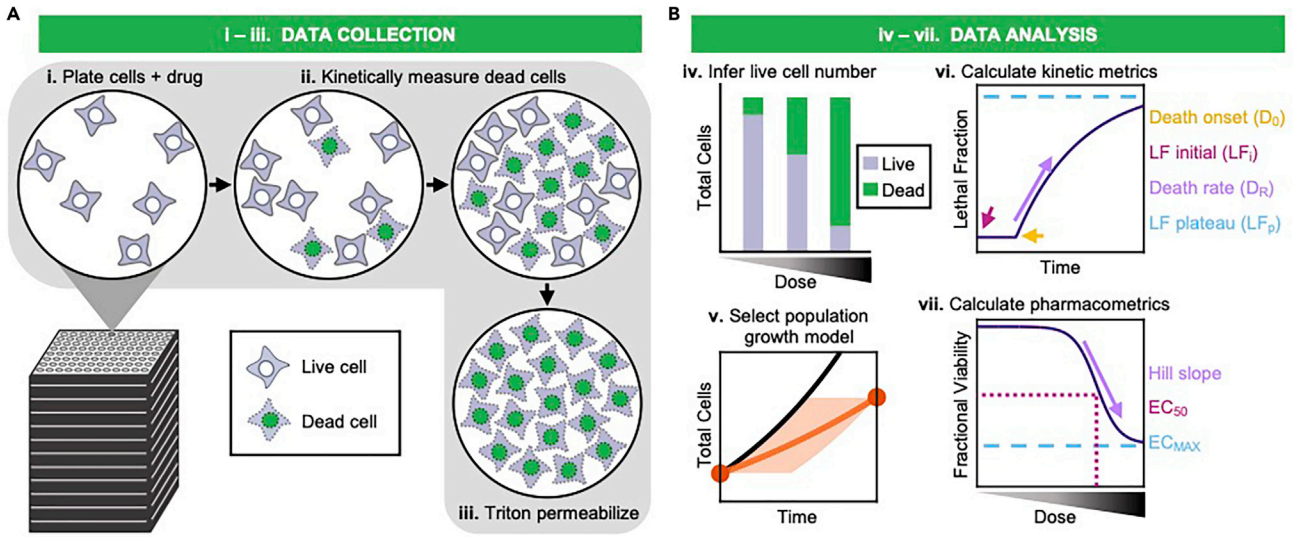

Figure 3. Overview of the FLICK assay

(A) Schematic of steps for data collection using FLICK.

(B) Schematic of steps for analysis of data generated using FLICK.

16. After the final measurement, add 10 μL of 1% permeabilization buffer to each well. Lyse the plate for > 2 h at 37°C and 5% CO_2
    a. Once all wells have been fully lysed, take a final fluorescence measurement. This final measurement is proportional to the total cell count at the end of the assay.

⚠ CRITICAL: Before taking the total cell count measurement, check the plate for complete lysis under a microscope. See Before you begin, step 8, and Figures 1B and 1C for more details.

EXPECTED OUTCOMES

The FLICK method is designed to provide a specific measurement of cell death, rather than an indirect surrogate of cell viability. Other assays that are specific to cell death generally use time-lapse microscopy to track live and dead cells over time. In the FLICK method, this is achieved by direct measurement of the dead cell population over time, and a computational inference of the live cell population. Additionally, because FLICK does not require the use of any genetically encoded labels, this method can be used more flexibly, to quantify responses in diverse settings, including for primary cells.

The outlined procedure will generate temporally resolved fluorescence data that is proportional to the number of dead cells contained within a well (Figure 3A). Additionally, this protocol generates an experimentally determined measurement that is proportional to the total number of cells (e.g., live and dead) at the start and end of the assay. Total cell fluorescence at intermediate time points (i.e., any time point between the beginning and end of the assay) is computationally inferred using the experimentally observed cell fluorescence at the start and end of the assay as constraints (see Quantification and statistical analysis, Figure 3B). For all wells, fluorescence following endpoint permeabilization should be equal to- or higher than the T0 fluorescence (Figures 4A and 4B, see Troubleshooting section for additional details). For vehicle-treated wells, fluorescence measurements after endpoint permeabilization should be substantially higher than measurements taken at the end point of the assay prior to permeabilization, reflecting an expected low percentage of dead cells in the population (Figures 4B and 4C). Outcomes for drug-treated wells will vary depending on the concentration and mechanism of action (death, growth arrest, or a combination of death and arrest)

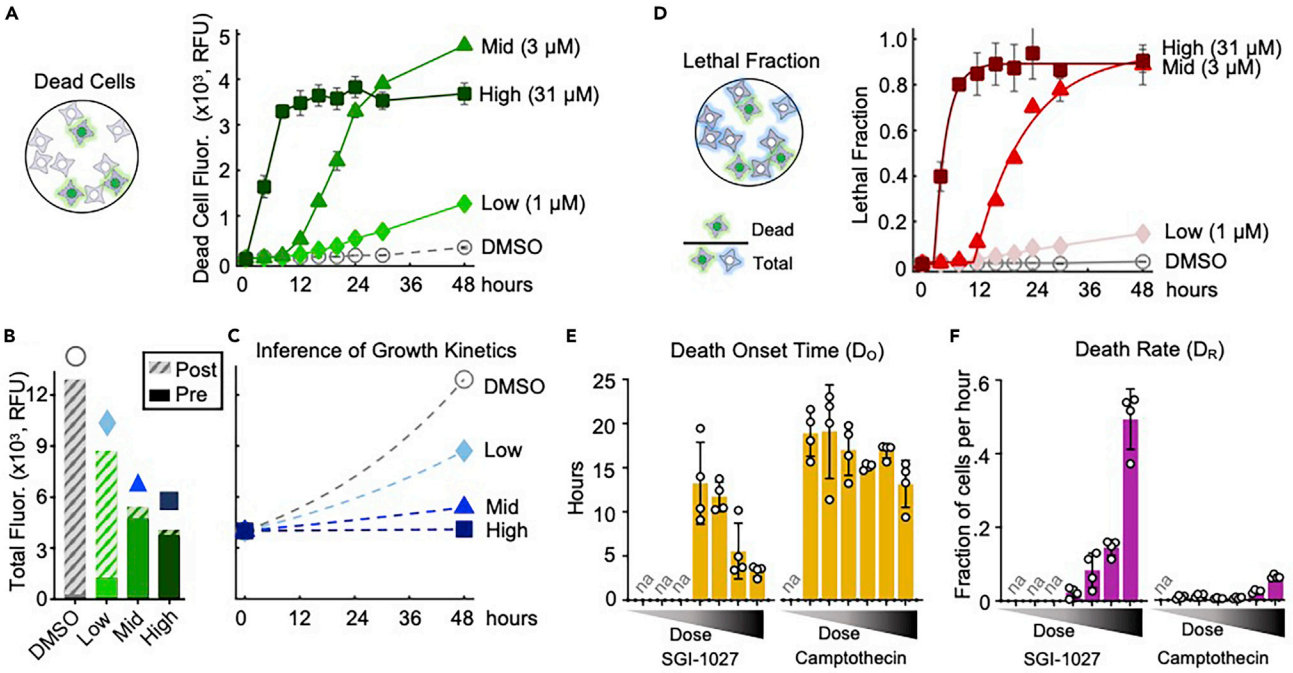

Figure 4. Kinetic analysis of drug response using the FLICK assay

Analysis of response to SGI-1027 at varied doses. Due to varied rates of death onset, SGI-1027 results in cell death that appears to peak at intermediate doses.

(A) Quantification of dead cell fluorescence over time.

(B) Quantification of live cell fluorescence at assay endpoint. The live cell fluorescence corresponds to the difference between the SYTOX fluorescence pre-Triton permeabilization (PRE) and post-Triton (POST) permeabilization.

(C) Population size at any intermediate time point is estimated based on the experimentally observed population size at the beginning and end of the assay.

(D) Lethal fraction (LF) kinetics for SGI-1027. Unlike dead cell fluorescence, LF reveals dose-dependent increase in drug efficacy.

(E and F) Kinetic parameters from the LED analysis. (E) Death onset time (D_O) at each tested dose of SGI-1027. Camptothecin shown for comparison. (F) Death rate (D_R) at each tested dose of SGI-1027. Camptothecin shown for comparison. For (A) and (D)–(F), data are mean ± SD of 4 biological replicates. For (F) and (G), dose ranges tested were half-log dilutions starting at 31.6 μM. If death was not significantly above baseline values at the assay endpoint, rates were not calculated (n.a., not applicable).

(Schwartz et al., 2020). Death-inducing drugs should cause an increase in the fluorescence signal over time and may plateau at later time points. Alternatively, drugs that primarily induce growth arrest may not cause an increase in SYTOX fluorescence, but a noticeable decrease in the total cell fluorescence after permeabilization should be observed.

Drug-induced cell death can be observed directly from the SYTOX fluorescent signal, which is proportional to the number of dead cells; however, this signal alone should not be used to evaluate drug response, due to the lack of insight about population size (Forcina et al., 2017). The peculiar concentration dependence of SGI-1027 provides an example of unreliable insights derived from counting dead cells alone, when population size is not also considered. SGI-1027 is a DNA methyl-transferase inhibitor which leads to both growth arrest and cell death, but with varied timing and varied intensity, depending on the dose used. At low concentrations (1 μM), SGI-1027 causes low levels of cell death (Figure 4A). At these low concentrations, however, the total cell count at the end of the experiment is reduced compared to the vehicle control, revealing a modest anti-proliferative effect at this concentration (Figures 4B and 4C). At higher concentrations (3.16 and 31.6 μM), all cells are killed by the end of the assay; however, the data do not follow an expected dose-dependent response. Instead, SGI-1027 at 3.16 μM kills a larger total number of cells than at a higher dose of 31.6 μM (Figure 4A). This discrepancy is caused by the fact that exposure to 31.6 μM SGI-1027 results in a very fast onset time of death (D_O) and rate of death (D_R), essentially killing all plated cells before any cell division can

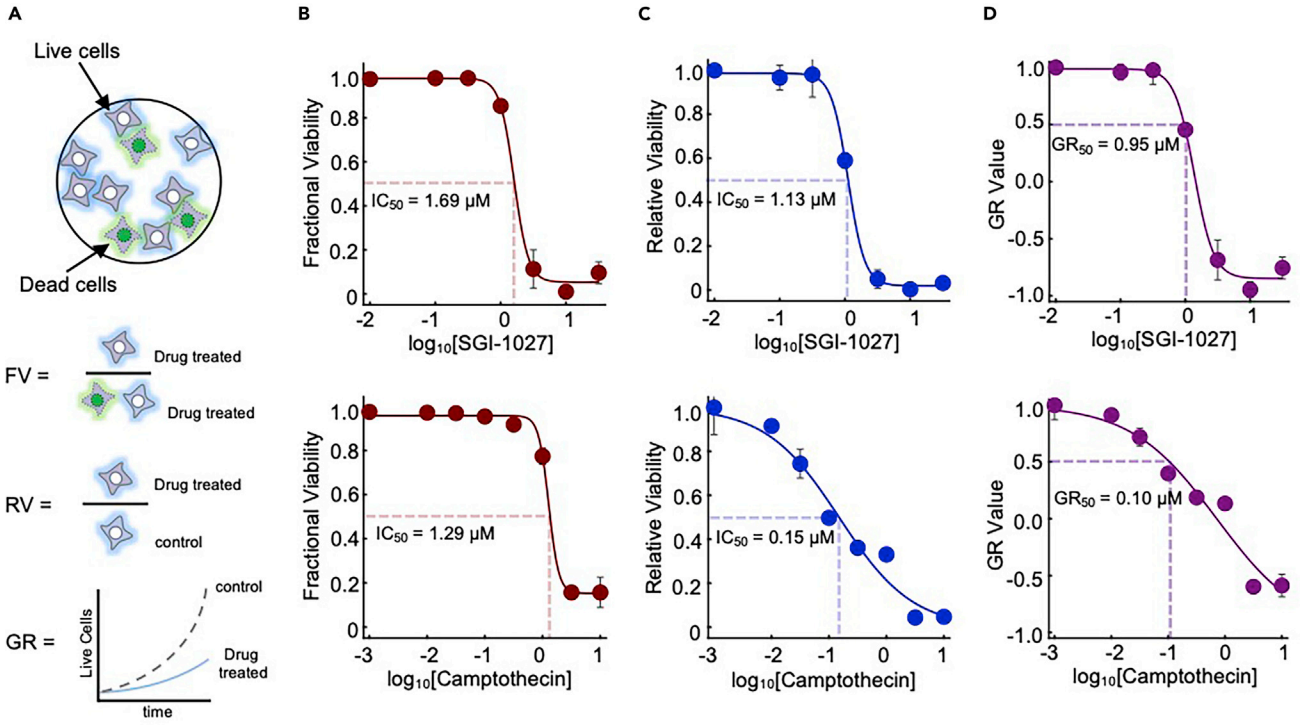

Figure 5. Pharmacological analyses of drug response

Comparison of common pharmaco-metrics.

(A) Definitions for fractional viability (FV), relative viability (RV), and normalized growth rate inhibition (GR). FV is the fraction of a cell population that is alive. RV is the size of the drug-treated cell population, compared to the vehicle control population. GR is the relative population growth rate of drug-treated cells, compared to control.

(B–D) Sample data for response to SGI-1027 (top) or Camptothecin (bottom) in U2-OS cells. (B) FV. (C) RV (D) GR. For each panel in (B)–(D), the IC_{50}, or equivalent, is shown. IC_{50} refers to the dose associated with 50% reduction from the starting value. Curve fitting generates the EC_{50} parameter, which refers to the dose of the observed half-maximal response. Data are mean ± SD of biological replicates.

occur. Computing the drug-induced lethal fraction – which considers both the number of dead cells and the population size – reveals a more reliable insight about the drug response, including the dose-dependent variation in death onset time (Figures 4D–4F). Additionally, lethal fraction or its inverse, fractional viability, also reveals a more typical dose-dependent increase in cell death (Figure 4D, 5A, and 5B).

Lethal fraction or fractional viability produce reliable insights regarding the degree of drug-induced cell killing. However, these measures do not capture drug-induced changes in the population size. Alternatively, relative viability, the most commonly used measurement for evaluating drug sensitivity, ignores the degree of cell killing, instead reporting only changes in population size (Figures 5A and C). Importantly, because this protocol facilitates quantification of both live and dead cells, these methods can be used to quantify any commonly used drug response metric, including relative viability or the normalized growth rate inhibition value (GR) (Figures 5A and 5D). Drugs vary in the degree to which they activate cell death versus inhibit growth (Schwartz et al., 2020). For drugs like SGI-1027, that primarily activate death without strongly inhibiting cell proliferation, FV, RV, and GR metrics will produce very similar insights (Figures 4B–4D). Alternatively, other drugs like camptothecin activate cell death at high doses, but also strongly inhibit cell proliferation, even at low doses. For these drugs, FV, RV, and GR metrics tend to produce different insights (Figures 4B–4D). Each of these metrics can be appropriate, depending on the focus of a study, but each metric produces unique and incomplete insights. Additionally, using data generated from this protocol, the complementary insights derived from cell death-focused or population size-focused

Table 2. Fluorescence of dead cells (FD_t)

		SYTOX fluorescence (relative fluor. units, RFU)						
Camptothecin dose (μM)	Replicate #	FD_0	FD_{16}	FD_{20}	FD_{24}	FD_{40}	FD_{44}	FD_{48}
0	1	234	236	251	247	325	332	354
0.01	1	239	273	264	269	341	349	405
0.0316	1	253	252	258	267	335	346	351
0.1	1	224	253	259	261	387	445	388
0.316	1	238	265	277	299	407	471	501
1	1	242	371	593	856	1,058	1,222	1,364
3.16	1	257	647	1,324	1,765	2,630	3,169	3,630
10	1	239	502	1,142	1,862	3,201	3,457	3,820
0	2	226	236	253	258	347	385	439
0.01	2	230	279	246	257	344	388	429
0.0316	2	246	254	251	266	347	361	389
0.1	2	221	232	245	253	304	340	358
0.316	2	267	268	282	282	392	550	515
1	2	250	352	567	776	1,128	1,393	1,674
3.16	2	238	600	1,255	1,688	2,659	3,253	3,819
10	2	262	604	1,317	2,154	3,308	3,836	4,131

pharmacometrics can be integrated by calculating drug GRADE (Schwartz et al., 2020). GRADE analysis reveals the degree of drug-induced cell death versus drug-induced proliferative arrest induced by a given drug. Other common assays generate signals proportional to metabolic activity (CellTiter-Glo, MTT, Alomar Blue). Unlike FLICK, these other assays do not provide specific information on the degree of drug-induced cell death, and can only be used to score relative viability.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantify the fluorescence associated with dead and live cells over time

1. Determine the SYTOX fluorescence of dead cells (FD_t) at each time point for each condition (Figure 4A)
   a. SYTOX fluorescence is proportional to the number of dead cells and should be measured immediately after the assay start, followed by measurements at any additional timepoints (t) of interest (Table 2).

2. Model the population growth over time (Figures 4B and 4C).
   a. After the last time point has been taken, determine the SYTOX fluorescence of total cells (FT) at the end of the experiment by measuring the fluorescence following permeabilization with Triton (Figure 4B and Table 3, FT_{48}).

   Note: Fluorescence values following Triton permeabilization are proportional to the total number of cells (live + dead).

   b. Determine the SYTOX fluorescence following Triton permeabilization for the T0 Control plate (FT_0).
      i. An average starting value (FTM_0) should be determined by calculating the 50% trimmed mean from the fluorescence of permeabilized cells (Table 4). The trimmed mean refers to the mean of the data, following removal of the highest and lowest outliers.
         - In MATLAB, use the built-in function "trimmean" (i.e., trimmean(x,50)).
         - In Excel or similar, sort the T0 data and remove the top and bottom 25%. Compute the mean of the remaining 50%.

   Note: Computing an average starting fluorescence value is done here for robustness, as well-to-well variations are likely to fluctuate from plate to plate. Computing this average from a

Table 3. Total cell fluorescence at assay endpoint (FT_{48})

		SYTOX fluorescence of total cells (RFU)						
Camptothecin dose (μM)	Rep. #	FTM_0	FT_{16}	FT_{20}	FT_{24}	FT_{40}	FT_{44}	FT_{48}
0	1	–	–	–	–	–	–	14,747
0.01	1	–	–	–	–	–	–	14,713
0.0316	1	–	–	–	–	–	–	12,829
0.1	1	–	–	–	–	–	–	8,507
0.316	1	–	–	–	–	–	–	6,411
1	1	–	–	–	–	–	–	6,828
3.16	1	–	–	–	–	–	–	4,376
10	1	–	–	–	–	–	–	4,809
0	2	–	–	–	–	–	–	17,605
0.01	2	–	–	–	–	–	–	15,027
0.0316	2	–	–	–	–	–	–	11,393
0.1	2	–	–	–	–	–	–	7,935
0.316	2	–	–	–	–	–	–	6,011
1	2	–	–	–	–	–	–	6,648
3.16	2	–	–	–	–	–	–	4,462
10	2	–	–	–	–	–	–	4,636

trimmed portion of the data may reduce the influence of single outlier wells that may exist for technical reasons (incomplete lysis, error in plating, etc.). A similar method is used in other studies (Hafner et al., 2016).

c. For each condition, fit the starting (FTM_0) and final (FT_{48}) total cell fluorescence values to an exponential curve:

$$FT(t) = ae^{(kt)}$$

t = time point
k = growth rate per hour
a = initial starting population

i. In MATLAB, this can be performed using the built-in function "fit" and the fit type "exp1."
ii. In PRISM, this can be performed using the nonlinear regression curve fitting package, with the exponential growth equation.
iii. In excel, this can be performed by generating a signal vs. time scatter plot, and adding an exponential trendline

d. Estimate the total fluorescence at each intermediate time point (FT_t) by solving the above equation at all desired time points (t), once the a and k parameters have been solved from the curve fitting (Figure 4C and Table 5).

Note: This estimate is based on the assumption of exponential population growth at uniform rate. Population growth over time in the absence of drug is likely to be approximately exponential. In the presence of drug, the population growth dynamics may not be exponential and may not be uniform over time. Our simulations demonstrate that the accuracy of lethal fraction kinetics do not depend on the validity of this assumption, as lethal fraction kinetics computed using this method were similar under many different assumed growth models. Additionally, lethal fraction kinetics computed using this method were similar to those experimentally

Table 4. Fluorescence of total population at assay start (FT_0)

	SYTOX fluorescence of total population at T0 (RFU)							
FT_0	3,197	3,470	3,475	3,685	3,700	3,883	3,927	3,988
	…	…	…	…	…	…	…	…
	3,994	4,316	4,320	4,519	5,021	5,814	6,359	6,477
50% trimmed mean (FTM_0)	4,081							

Table 5. Fluorescence of the total population (FT_t)

		SYTOX fluorescence of the total population (RFU)						
Camptothecin dose (μM)	Rep. #	FTM_0	FT_{16}	FT_{20}	FT_{24}	FT_{40}	FT_{44}	FT_{48}
0	1	4,081	6,262	6,970	7,758	11,905	13,250	14,747
0.01	1	4,081	6,258	6,963	7,749	11,882	13,222	14,713
0.0316	1	4,081	5,978	6,577	7,236	10,600	11,661	12,829
0.1	1	4,081	5,213	5,542	5,892	7,527	8,002	8,507
0.316	1	4,081	4,744	4,926	5,115	5,946	6,174	6,411
1	1	4,081	4,845	5,057	5,279	6,267	6,541	6,828
3.16	1	4,081	4,177	4,201	4,226	4,325	4,351	4,376
10	1	4,081	4,310	4,370	4,430	4,679	4,744	4,809
0	2	4,081	6,643	7,504	8,476	13,798	15,586	17,605
0.01	2	4,081	6,302	7,025	7,831	12,093	13,480	15,027
0.0316	2	4,081	5,746	6,259	6,819	9,601	10,459	11,393
0.1	2	4,081	5,094	5,384	5,690	7,103	7,507	7,935
0.316	2	4,081	4,643	4,795	4,953	5,635	5,820	6,011
1	2	4,081	4,802	5,001	5,209	6,129	6,383	6,648
3.16	2	4,081	4,204	4,236	4,267	4,396	4,429	4,462
10	2	4,081	4,258	4,304	4,350	4,538	4,587	4,636

calculated from time-lapse microscopy. This robustness depends on the length of the assay, as in short-term assays (48–72 h; effectively 2–3 population doublings), many different growth models produce a similar inference of live cell numbers or death kinetics. For longer assays, other growth models may provide more accurate estimates, including growth models with non-uniform rates over time (Richards, et al. 2020). See comment in Limitations below.

e. Subtract the measured fluorescence of dead cells (Table 2) from the total cell fluorescence generated in Table 5. The remaining fluorescence is proportional to the size of the live population at each time point (Table 6).

$$FL(t)_x = FT_{t,x} - FD_{t,x}$$

FT = fluorescence of total cells (Table 5)
FD = fluorescence of dead cells (Table 2)
t = a given timepoint
x = a given drug concentration

Note: This simple subtraction (fluorescence of the total population – fluorescence of the dead population) should be proportional to the number of live cells. The accuracy of this inference depends on the linearity of the SYTOX fluorescence signal with cell number (Before you begin, steps 10 and 11, Figure 1D).

Calculate lethal fraction (LF) kinetics

3. Calculate lethal fraction kinetics (Figure 4D).
   a. Calculate lethal fraction (LF) for each condition/timepoint (Table 7):

$$LF(t)_x = \frac{FD_{t,x}}{FD_{t,x} + FL_{t,x}}$$

FD = dead cell fluorescence (from Table 2)
FL = live cell fluorescence (from Table 6)
t = a given time point
x = a given drug concentration

   b. For each dose of drug, determine response kinetics using a Lag Exponential Death (LED) equation (Forcina et al., 2017) (Table 8):

$$LF(t) = LF_i + \left(LF_P - LF_0\right) * \left(1 - e^{-D_R(t-D_O)}\right)$$

Table 6. Fluorescence of live cells (FL_t)

		SYTOX fluorescence of live cells (RFU)						
Camptothecin dose (μM)	Rep. #	FL_0	FL_{16}	FL_{20}	FL_{24}	FL_{40}	FL_{44}	FL_{48}
0	1	3,847	6,026	6,719	7,511	11,580	12,918	14,393
0.01	1	3,842	5,985	6,699	7,480	11,541	12,873	14,308
0.0316	1	3,828	5,726	6,319	6,969	10,265	11,315	12,478
0.1	1	3,857	4,960	5,283	5,631	7,140	7,557	8,119
0.316	1	3,843	4,479	4,649	4,816	5,539	5,703	5,910
1	1	3,839	4,474	4,464	4,423	5,209	5,319	5,464
3.16	1	3,824	3,530	2,877	2,461	1,695	1,182	746
10	1	3,842	3,808	3,228	2,568	1,478	1,287	989
0	2	3,855	6,407	7,251	8,218	13,451	15,201	17,166
0.01	2	3,851	6,023	6,779	7,574	11,749	13,092	14,598
0.0316	2	3,835	5,492	6,008	6,553	9,254	10,098	11,004
0.1	2	3,860	4,862	5,139	5,437	6,799	7,167	7,577
0.316	2	3,814	4,375	4,513	4,671	5,243	5,270	5,496
1	2	3,831	4,450	4,434	4,433	5,001	4,990	4,974
3.16	2	3,843	3,604	2,981	2,579	1,737	1,176	643
10	2	3,819	3,654	2,987	2,196	1,230	751	505

LF_i = initial lethal fraction prior to death onset, unconstrained
LF_p = lethal fraction plateau, constrained by $LF_p < 1$
D_O = onset time of death, constrained by $0 < D_O <$ final timepoint
D_R = maximum rate of death, constrained by $D_R < 2$
The four kinetic parameters of the LED model should be determined using a least squares regression based curve fitting.

i. In MATLAB, this can be performed using the built-in function "fit." See associated GitHub repository to access a custom built function.
ii. In PRISM, LED parameters can be solved using the "plateau followed by one-phase association" function in the nonlinear regression, exponential analysis package, with manually constrained death onset time (X0 in PRISM).

Note: the LED equation is useful for determining the kinetics of cell death, namely through the D_O and D_R parameters. These features are not observable from end point data, and differ across drugs and drug classes (Inde et al., 2020). SGI-1027 has a dose-dependent decrease in D_O whereas Camptothecin has more stable D_O across doses (Figure 4E). Both SGI-1027 and Camptothecin result in near complete cell killing at high doses, but the death occurs at very different rates (Figure 4F).

c. For drugs/doses that do not induce significant levels of cell death (see Table 8, *), kinetic terms are unreliable. These data are instead fit to a linear model with slope = 0 (Table 9):

$$LF(t) = 0(t) + LF_i$$

LF_i = initial lethal fraction prior to death onset, unconstrained

i. Lack of drug-induced cell death can be determined based on the error among replicates (i.e., no significant change from LF_i over the time course). Alternatively, non-killing drugs can be defined as those for which LF_{max} values are not 2× greater than LF_i.
ii. In MATLAB, curve fitting can be performed using the built-in function "fit," defining the fit-type as "poly1."
iii. In PRISM or Excel, data can be fit using linear regression.

Table 7. Lethal fraction (LF_t)

		Lethal fraction						
Camptothecin dose (μM)	Rep. #	LF_0	LF_{16}	LF_{20}	LF_{24}	LF_{40}	LF_{44}	LF_{48}
0	1	0.0573	0.0377	0.0360	0.0318	0.0273	0.0251	0.0240
0.01	1	0.0586	0.0436	0.0379	0.0347	0.0287	0.0264	0.0275
0.0316	1	0.0620	0.0422	0.0392	0.0369	0.0316	0.0297	0.0274
0.1	1	0.0549	0.0485	0.0467	0.0443	0.0514	0.0556	0.0456
0.316	1	0.0583	0.0559	0.0562	0.0585	0.0684	0.0763	0.0781
1	1	0.0593	0.0766	0.1173	0.1622	0.1688	0.1868	0.1998
3.16	1	0.0630	0.1549	0.3151	0.4177	0.6080	0.7284	0.8295
10	1	0.0586	0.1165	0.2613	0.4203	0.6841	0.7288	0.7943
0	2	0.0554	0.0355	0.0337	0.0304	0.0251	0.0247	0.0249
0.01	2	0.0564	0.0443	0.0350	0.0328	0.0284	0.0288	0.0285
0.0316	2	0.0603	0.0442	0.0401	0.0390	0.0361	0.0345	0.0341
0.1	2	0.0542	0.0455	0.0455	0.0445	0.0428	0.0453	0.0451
0.316	2	0.0654	0.0577	0.0588	0.0569	0.0696	0.0945	0.0857
1	2	0.0613	0.0733	0.1134	0.1490	0.1841	0.2182	0.2518
3.16	2	0.0583	0.1427	0.2963	0.3956	0.6049	0.7345	0.8559
10	2	0.0642	0.1418	0.3060	0.4952	0.7289	0.8363	0.8911

Calculate pharmacological dose-responses using fractional viability (FV), relative viability (RV), or normalized growth rate adjusted values (GR)

4. Calculate fractional viability (FV, Figures 5A and 5B)

Note: FV is a common measure of drug response for assays that count both live and dead cells (flow cytometry, quantitative microscopy, etc.). Unlike RV, FV does not report changes in population size, but rather the proportion of the population that is alive or dead. FV is the inverse of LF (FV = 1 − LF).

a. Calculate fractional viability (FV) for each condition/timepoint (Table 10):

$$FV(t)_x = 1 - LF_{t,x}$$

LF = lethal fraction (from Table 6)
t = a given time point
x = a given drug concentration

b. Determine pharmacological parameters for FV values by curve fitting, using the same 4-parameter logistic regression as for RV, above in Quantification and statistical analysis step 4b.

5. Calculate relative viability (RV, Figures 5A and 5C).

Note: RV is a common measure of drug response used for most assays that rely on measures of metabolic activity (CellTiter-Glo, MTT, Alamar Blue, etc.). This measure reports the drug-induced change in population size, compared to untreated or vehicle-treated populations.

a. Calculate relative viability (RV) for each condition/timepoint (Table 11).

i. Replicates of the control condition are averaged before calculating RV:

$$RV(t)_x = \frac{FL_{t,x}}{FL_{t,0}}$$

FL = modeled live cell fluorescence (from Table 6)
t = a given time point
x = a given drug concentration

b. Model RV values using a 4-parameter logistic regression (at any timepoint but most commonly using data from the assay endpoint):

Table 8. Lag exponential death (LED) fitted parameters

		Lethal fraction							
Camptothecin dose (μM)	Rep. #	LF_0	…	LF_{48}	LF_i	LF_p	D_O	D_R	LF_{max}
0	1	0.0573	…	0.0240	0.056	0.022	0.953	0.054	0.057*
0	2	0.0554	…	0.0249					
0.01	1	0.0586	…	0.0275	0.057	0.028	11.374	0.133	0.059*
0.01	2	0.0564	…	0.0285					
0.0316	1	0.0620	…	0.0274	0.061	0.030	1.841	0.060	0.062*
0.0316	2	0.0603	…	0.0341					
0.1	1	0.0549	…	0.0456	0.048	0.046	34.480	0.014	0.056*
0.1	2	0.0542	…	0.0451					
0.316	1	0.0583	…	0.0781	0.059	0.084	39.558	1.176	0.095*
0.316	2	0.0654	…	0.0857					
1	1	0.0593	…	0.1998	0.060	0.219	14.586	0.081	0.252
1	2	0.0613	…	0.2518					
3.16	1	0.0630	…	0.8295	0.061	1.000	13.239	0.041	0.856
3.16	2	0.0583	…	0.8559					
10	1	0.0586	…	0.7943	0.061	0.957	14.547	0.056	0.891
10	2	0.0642	…	0.8911					

$$RV(x) = E_{max} + \frac{RV_0 - E_{max}}{1 + 10^{((x - EC_{50}) \times hill)}}$$

x = dose in log10 scale
E_{max} = maximum effect (e.g., bottom of curve)
RV_0 = relative viability in absence of drug (e.g., top of curve)
EC_{50} = dose in log scale corresponding to the mid-point of the sigmoidal curve (inflection point)
h = Hill coefficient
The four parameters of the logistic regression model should be determined using a least squares regression based curve fitting.

i. In MATLAB, this can be performed using the built-in function "fit," defining the fit type using the equation listed above. See associated GitHub repository to access a custom built function.
ii. In PRISM, this can be performed using the "log(inhibitor) vs. response – variable slope" analysis function.

Note: This equation requires a log-transformation of the dose values and cannot accept a dose of "0." These doses can be omitted from the fit or set to a value 10× lower than the lowest dose.

6. Calculate the normalized growth rate inhibition value (GR, Figures 5A and 5D).

Note: GR reports the drug response in terms of the growth rate of drug-treated cells, compared to the untreated growth rate. This approach normalizes for differences in the apparent drug response that may be due to differences in assay length or differences in growth rates between cells of interest. GR calculations are described in detail in Hafner et al. 2016.

a. Calculate the average fluorescence of live cells from the replicates of untreated control wells at the assay start ($FL_{0,ctrl}$, see Table 5) (Table 12).
b. Calculate the average fluorescence of live cells from the replicates of the untreated control at the timepoint of interest ($FL_{t,ctrl}$, see Table 6). An example calculating the mean fluorescence of live cells at 48 h is shown in Table 13.

Table 9. Lag exponential death (LED) fitted parameters, linear model (flat fit) included

		Lethal fraction							
Camptothecin dose (μM)	Rep. #	LF_0	…	LF_{48}	LF_i	LF_P	D_O	D_R	LF_{max}
0	1	0.0573	…	0.0240	0.034	0	48	0	0.057
0	2	0.0554	…	0.0249					
0.01	1	0.0586	…	0.0275	0.037	0	48	0	0.059
0.01	2	0.0564	…	0.0285					
0.0316	1	0.0620	…	0.0274	0.040	0	48	0	0.062
0.0316	2	0.0603	…	0.0341					
0.1	1	0.0549	…	0.0456	0.048	0	48	0	0.056
0.1	2	0.0542	…	0.0451					
0.316	1	0.0583	…	0.0781	0.067	0	48	0	0.095
0.316	2	0.0654	…	0.0857					
1	1	0.0593	…	0.1998	0.060	0.219	14.586	0.081	0.252
1	2	0.0613	…	0.2518					
3.16	1	0.0630	…	0.8295	0.061	1.000	13.239	0.041	0.856
3.16	2	0.0583	…	0.8559					
10	1	0.0586	…	0.7943	0.061	0.957	14.547	0.056	0.891
10	2	0.0642	…	0.8911					

c. Determine the fluorescence contributed by the proportion of the population that was still alive at each timepoint in each of the treated conditions at the assay start ($FL_{0,d}$) and any timepoint of interest ($FL_{t,d}$) (see Table 6).

d. Calculate the GR value for each condition/timepoint (Table 14):

$$GR(t)_x = \frac{\log_2(FL_{t,x}/FL_{0,x})}{2^{\log_2(FLM_{t,ctrl}/FLM_{0,ctrl})} - 1}$$

FL = modeled live cell fluorescence (from Table 6)
FLM = mean fluorescence of live cells (from Tables 12 and 13)
t = a given timepoint
x = a given drug concentration

a. Determine pharmacological parameters for GR values by curve fitting, using the same 4-parameter logistic regression as for RV and FV, above in Quantification and statistical analysis step 4b.

LIMITATIONS

The FLICK assay provides a single unified platform for evaluating drug response kinetics, as well as determining a variety of pharmacological measures, such as relative viability, fractional viability, and GR values. These values can be obtained at any time point in the assay window, or at the assay end point. Additionally, because FLICK requires only one fluorescence channel to mark dead cells, this assay can be optimized to be multiplexed with markers of other cell fates or responses. This protocol has been optimized for adherent cell lines, but should be easily adapted to suspension cultures.

The FLICK assay has a few notable limitations. The use of a plate reader requires the user to manually move plates between the incubator and the reader. The lack of automation may limit throughput, although commercially available automated microscopes also generally accommodate only a small number of plates. More critically, FLICK requires that the user select time points for analysis. As discussed in this protocol (Fluorescence measurements, step 14) and in the Troubleshooting section, this may require some optimization depending on the kinetics of cell death observed for a given drug. An additional limitation is that the output of this assay is not cell number, but fluorescence values for a population of cells (i.e., a well of a plate). This value, however, is linearly proportional

Table 10. Fractional viability (FV_t)

		Fractional viability						
Camptothecin dose (μM)	Rep. #	FV_0	FV_{16}	FV_{20}	FV_{24}	FV_{40}	FV_{44}	FV_{48}
0	1	0.9427	0.9623	0.9640	0.9682	0.9727	0.9749	0.9760
0.01	1	0.9414	0.9564	0.9621	0.9653	0.9713	0.9736	0.9725
0.0316	1	0.9380	0.9578	0.9608	0.9631	0.9684	0.9703	0.9726
0.1	1	0.9451	0.9515	0.9533	0.9557	0.9486	0.9444	0.9544
0.316	1	0.9417	0.9441	0.9438	0.9415	0.9316	0.9237	0.9219
1	1	0.9407	0.9234	0.8827	0.8378	0.8312	0.8132	0.8002
3.16	1	0.9370	0.8451	0.6849	0.5823	0.3920	0.2716	0.1705
10	1	0.9414	0.8835	0.7387	0.5797	0.3159	0.2712	0.2057
0	2	0.9446	0.9645	0.9663	0.9696	0.9749	0.9753	0.9751
0.01	2	0.9436	0.9557	0.9650	0.9672	0.9716	0.9712	0.9715
0.0316	2	0.9397	0.9558	0.9599	0.9610	0.9639	0.9655	0.9659
0.1	2	0.9458	0.9545	0.9545	0.9555	0.9572	0.9547	0.9549
0.316	2	0.9346	0.9423	0.9412	0.9431	0.9304	0.9055	0.9143
1	2	0.9387	0.9267	0.8866	0.8510	0.8159	0.7818	0.7482
3.16	2	0.9417	0.8573	0.7037	0.6044	0.3951	0.2655	0.1441
10	2	0.9358	0.8582	0.6940	0.5048	0.2711	0.1637	0.1089

to the number of cells, so the number of cells can be estimated from fluorescence values by generating a standard curve, similar to the cell dilution used when calibrating SYTOX (see Before you begin, step 1).

Some drugs emit fluorescence in the same wavelength as SYTOX and cannot be quantified using this assay. In these situations, use of SYTOX variants that emit fluorescence in other wavelengths may be helpful. This assay is also unable to measure the response to drugs that interfere with SYTOX fluorescence or the ability of SYTOX to bind DNA. Lastly, the demarcation of dead cells by SYTOX requires both plasma membrane rupture and disruption of the nuclear envelope. These membranes require active maintenance, so SYTOX will mark dead cells regardless of the mechanism of cell death caused by the drug. These events, however, do not occur with the same efficiency for all types of cell death, which may affect the ability of SYTOX to accurately quantify dead cells.

Table 11. Relative viability (RV_t)

		Relative viability						
Camptothecin dose (μM)	Rep. #	RV_0	RV_{16}	RV_{20}	RV_{24}	RV_{40}	RV_{44}	RV_{48}
0	1	0.9990	0.9694	0.9619	0.9550	0.9252	0.9188	0.9121
0.01	1	0.9977	0.9626	0.9591	0.9511	0.9221	0.9156	0.9067
0.0316	1	0.9940	0.9211	0.9046	0.8861	0.8202	0.8048	0.7908
0.1	1	1.0016	0.7979	0.7564	0.7160	0.5705	0.5375	0.5145
0.316	1	0.9979	0.7205	0.6656	0.6124	0.4426	0.4057	0.3745
1	1	0.9969	0.7196	0.6391	0.5624	0.4162	0.3783	0.3463
3.16	1	0.9930	0.5678	0.4119	0.3129	0.1355	0.0840	0.0473
10	1	0.9977	0.6126	0.4621	0.3265	0.1181	0.0915	0.0627
0	2	1.0010	1.0306	1.0381	1.0450	1.0748	1.0812	1.0879
0.01	2	1.0000	0.9688	0.9705	0.9631	0.9387	0.9312	0.9251
0.0316	2	0.9958	0.8834	0.8602	0.8332	0.7394	0.7182	0.6974
0.1	2	1.0023	0.7820	0.7357	0.6914	0.5432	0.5098	0.4802
0.316	2	0.9904	0.7038	0.6462	0.5939	0.4189	0.3748	0.3483
1	2	0.9948	0.7158	0.6348	0.5636	0.3996	0.3549	0.3152
3.16	2	0.9979	0.5797	0.4267	0.3280	0.1388	0.0836	0.0407
10	2	0.9917	0.5878	0.4276	0.2792	0.0983	0.0534	0.0320

Table 12. Untreated control at assay start ($FL_{0,ctrl}$)

		Live cell fluorescence	
Camptothecin dose (μM)	Replicate #	$FL_{0,ctrl}$	$FLM_{0,ctrl}$
0	1	3,847	3,851
0	2	3,855	

Our kinetic inference of live and dead cells over time makes one critical assumption regarding the nature of growth for drug-treated populations. For any population growth that is observed over time, our approach assumes that growth was exponential at a constant rate over time. While approximately exponential growth is generally observed for healthy cells, the same is not necessarily true for cells experiencing drug-induced stress. By simulating responses using a spectrum of different growth models, we previously found that inferred response kinetics were stable regardless of which growth model was chosen (e.g., linear, sigmoidal, and non-uniform over time) (Richards et al., 2020). This overall robustness regardless of growth model depends on selection of a relatively short assay time (48–72 h; generally 2–3 population doublings). At longer time points, solutions derived from different growth assumptions will begin to diverge more substantially. In these situations, selection of a population growth model that more accurately mimics what is observed for drug-induced growth may be helpful.

Even at short assay times, our simulations did identify some situations that differ substantially from the uniform exponential growth rate assumption; however, these required very peculiar growth kinetics that were not ever observed experimentally. Furthermore, the kinetic data that result from an exponential growth assumption correlate well with data derived from single cell microscopy for a panel of diverse drugs. Nonetheless, in some situations the assumption of a constant exponential growth rate may produce incorrect inferences. Kinetic inference of total cells can become inaccurate for drugs that kill at very fast rates and with early onset times. Drugs with these features compromise our calculation because the cells have decayed too much by the assay endpoint to produce a SYTOX signal. These situations could be remedied by ending the assay at an earlier time point.

TROUBLESHOOTING

Problem

Final pre-Triton measurement is higher than the post-Triton measurement (steps 14–16).

Potential solution

In principle, using Triton to lyse remaining viable cells should always lead to stable or increased SYTOX signal. If lethal fraction is ~ 1, post-Triton measurements may be slightly lower or higher, just due to measurement noise. A post-Triton SYTOX signal that is substantially decreased from the pre-Triton measurement can result from agitating the plate and dislodging the dead cells which may settle in a portion of the well in which fluorescence readings are not captured. This issue can be remedied by allowing the dislodged cells to settle before reading the plate, or by repeating the experiment being careful not to shake or agitate the plate.

Problem

Nonlinear relationship between cell number and SYTOX fluorescence (Before you begin, step 11).

Table 13. Untreated control at timepoint of interest ($FL_{t,ctrl}$)

		Live cell fluorescence	
Camptothecin dose (μM)	Replicate #	$FL_{48,ctrl}$	$FLM_{48,ctrl}$
0	1	14,393	15,780
0	2	17,166	

Table 14. GR values (GR_t)

		GR index						
Camptothecin dose (μM)	Rep. #	GR_0	GR_{16}	GR_{20}	GR_{24}	GR_{40}	GR_{44}	GR_{48}
0	1	1	0.9148	0.9139	0.9145	0.9118	0.9124	0.9126
0.01	1	1	0.8992	0.9103	0.9093	0.9095	0.9102	0.9083
0.0316	1	1	0.7911	0.7922	0.7888	0.7862	0.7863	0.7873
0.1	1	1	0.4392	0.4424	0.4439	0.4364	0.4333	0.4417
0.316	1	1	0.2482	0.2482	0.2450	0.2399	0.2353	0.2356
1	1	1	0.2479	0.1920	0.1473	0.1966	0.1907	0.1894
3.16	1	1	−0.1093	−0.2818	−0.3481	−0.3802	−0.4667	−0.5521
10	1	1	−0.0126	−0.1835	−0.3237	−0.4298	−0.4432	−0.4867
0	2	1	1.0861	1.0864	1.0852	1.0854	1.0842	1.0835
0.01	2	1	0.9103	0.9315	0.9283	0.9270	0.9251	0.9249
0.0316	2	1	0.6817	0.6866	0.6821	0.6788	0.6790	0.6787
0.1	2	1	0.3964	0.3953	0.3947	0.3950	0.3927	0.3930
0.316	2	1	0.2198	0.2166	0.2174	0.2059	0.1890	0.1967
1	2	1	0.2420	0.1856	0.1521	0.1697	0.1520	0.1369
3.16	2	1	−0.0886	−0.2561	−0.3210	−0.3731	−0.4694	−0.5846
10	2	1	−0.0618	−0.2488	−0.4157	−0.4863	−0.5813	−0.6300

Potential solution

Assuming all signals are in the linear range (i.e., no readings of "INF," "NaN," "error," etc.), non-linearity can be caused by incomplete permeabilization. Visually inspect the plate to see if Triton permeabilization has caused rupture of all cells. Alternatively, increase the Triton permeabilization time.

Problem

Lethal fraction decreases over time (step 14).

Potential solution

Lethal fraction measurements generally increase over time until reaching a stable plateau. In rare cases, LF% increases rapidly before slowly decreasing to a lower plateau. This can occur either due to the emergence of a drug resistant clone or due to degradation of the SYTOX signal over time. These two possibilities can be distinguished by directly measuring live cell kinetics over time using the STACK assay (Forcina et al, 2017).

Problem

Inaccurate inference of death kinetics when comparing to other assays (Quantification and statistical analysis, step 3).

Potential solution

In some situations, users may want to use kinetic data derived from this approach to parameterize dose/time selection for other experiments, for instance, finding time points before or after the onset of cell death. These inferred times can be inaccurate if the population growth varies significantly from the exponential growth model used. This is particularly an issue if growth in drug-treated conditions has three characteristics: 1) very non-uniform growth over time, 2) abrupt transition from growing to non-growing, and 3) transitions from growing to non-growing are unrelated to the death onset time. In the absence of these three features, inferred kinetic rates from this method should be accurate even if the population growth is non-exponential. In rare situations where these issues occur, potential solutions are to: shorten the assay length to minimize differences between varied growth assumptions, compute death kinetics using a

different growth model, or use the STACK assay to directly observe death kinetics (Forcina, et al. 2017).

Problem

Unreliable death onset time (Quantification and statistical analysis, step 3).

Potential solution

Selection of times to measure SYTOX fluorescence affects the accuracy of the death onset calculation. The inferred death onset time will vary substantially between experiments if it is not appropriately constrained by death observed at the selected time points. A solution to this is to optimize the selection of time points or to add more time points. The inference of death onset time will be improved if death levels are sampled at ~3 consecutive time points, spaced 2–4 h apart, in the exponentially increasing phase of death, and in the plateau phase of death.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Michael Lee (michael.lee@umassmed.edu).

Materials availability

This protocol uses only materials and reagents that are commercially available.

Data and code availability

Sample datasets and code used for analyzing data using this protocol are available in a GitHub repositories (MJLee-Lab/fitGrowth; MJLee-Lab/fitLED; MJLee-Lab/fit_via).

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AUTHOR CONTRIBUTIONS

This protocol was conceived by R.R. and M.J.L. Methodology was developed, tested, and refined by R.R. Simulations to test the robustness of the growth assumptions were performed by M.E.H. Data for this manuscript were collected by M.E.H. The manuscript was written and edited by R.R., M.E.H., and M.J.L. Funding acquisition and supervision were performed by M.J.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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