FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 67768
Scriptwriter Name: Debopriya Sadhukhan
Project Page Link: https://review.jove.com/account/file-uploader?src=20669288

Title: Comprehensive Analysis of Drug Response using the FLICK Assay

Authors and Affiliations:

Gavin A. Birdsall, Michael J. Lee

Department of Systems Biology, UMass Chan Medical School

Corresponding Authors:

Michael J. Lee (Michael.lee@umassmed.edu)

Email Addresses for All Authors:

Gavin A. Birdsall (Gavin.birdsall@umassmed.edu)
Michael J. Lee (Michael.lee@umassmed.edu)

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   Can you record movies/images using your own microscope camera?

   Yes

   Nikon Eclipse TS100 (light microscope)

   If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

   2.3.1

   2.3.2

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 32
Number of Shots: 54

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

REQUIRED:

1.1. **Michael Lee:** This research focuses on understanding how anti-cancer drugs function. The primary goal is to investigate how these drugs activate cell death.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 2B, 2C, 2E.*

What are the current experimental challenges?

1.2. **Michael Lee:** Methods for evaluating drug sensitivity quantify the number of cells but do not indicate whether the cells are dying, the extent of cell death, or which death mechanisms are activated.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What research gap are you addressing with your protocol?

1.3. **Gavin Birdsall:** This protocol allows for precise calculations of drug-induced growth and death rates, which cannot be achieved by studying only population size, as is common with other assays.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 2I.*

What advantage does your protocol offer compared to other techniques?

1.4. **Gavin Birdsall:** FLICK scores both live and dead cells with equal precision within the same assay, with no limitations to the types of cells or forms of cell death that can be studied.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.5.1.*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Selection and Calibration of DNA Stain

Demonstrator: Gavin Birdsall

2.1. To begin, plate the desired number of cells in each well of a 96-well, optical bottom, black-walled plate **[1]**. Add 100 microliters of complete medium and allow the cells to adhere to the plate overnight **[2]**.

2.1.1. WIDE: Talent pipetting cells into the wells of a black-walled 96-well plate.

2.1.2. Talent pipetting 100 microliters of complete medium into the wells.

2.2. Add 10 microliters of 1.5 percent Triton-X solution to each well containing the plated cells without mixing **[1]**.

2.2.1. Talent adding 10 microliters of Triton-X solution to each well using a pipette.

2.3. Observe cell morphologies under a light microscope using a 10x *(ten-ex)* objective and inspect cells every hour **[1]** until cell bodies are no longer visible **[2]**.

2.3.1. SCOPE: 67768_screenshot_3.PNG

2.3.2. SCOPE: 67768_screenshot_4.PNG

2.4. For each concentration to be tested, plate 40,000 cells in 180 microliters of cell culture medium in triplicate along the leftmost column of a 96-well, optical bottom, black-walled plate **[1]**. Add 90 microliters of medium to the remaining wells **[2]**.

2.4.1. Talent plating cells in cell culture medium using a pipette into the leftmost column of the 96-well plate.

2.4.2. Talent adding 90 microliters of cell culture medium to the other wells.

2.5. Using a multichannel pipette, transfer 90 microliters from the leftmost column into the adjacent column to the right to create a 1:2 *(one to two)* serial dilution **[1]**. Pipette up and down 15 times to mix **[2-TXT]**.

2.5.1. Talent using a multichannel pipette to transfer 90 microliters from the leftmost column into the adjacent column to the right. NOTE: 2.5.1. and 2.5.2 are combined.

2.5.2. Talent pipetting up and down in the second column to mix. **TXT: Repeat serial dilution from column 2 onward, moving rightward; Continue until the second-to-last column**

2.6. For the second-to-last column, remove 90 microliters so that all wells contain 90 microliters of medium with a varied number of cells **[1]**.

2.6.1. Talent removing 90 microliters from the second-to-last column using a pipette.

2.7. Add 10 microliters of 10x DNA dye to each well of the 96-well plate **[1]**. Then, add 10 microliters of 1.5 percent Triton-X solution to permeabilize the cells **[2-TXT]**.

2.7.1. Talent pipetting 10 microliters of DNA dye into each well.

2.7.2. Talent adding 10 microliters of Triton-X solution to each well. **TXT: Incubate cells in this solution for the optimal time**

2.8. To measure the fluorescence intensities, load the plate into a fluorescence plate reader **[1]**, adjust excitation and emission wavelengths, and digital gain depending on the plate reader used **[2]**, and measure fluorescence intensities across the cell titration plate **[3]**. Quantify fluorescence over a range of acquisition settings **[4]**.

2.8.1. Talent loading the plate into a fluorescence plate reader.

2.8.2. Talent adjusting excitation and emission wavelengths, and digital gain on the plate reader software. *Videographer: Please make sure the computer screen is clearly visible in the frame.* NOTE: This shot was slightly modified during the shoot.

2.8.3. SCREEN: 67768_screenshot_5.mp4 00:01-00:20, 01:00-01:08.

2.8.4. SCREEN: 67768_screenshot_6.mp4 00:01-00:16, 00:30-00:45, 01:28-01:34, 02:42-02:45. *Video Editor: Speed up the video as required.*

2.9. Subtract the average signal of the column containing no cells to remove the background signal from each measurement **[1]**.

2.9.1. SCREEN: 67768_screenshot_1.mp4 00:00-00:11.

2.10. Plot the cell number against the fluorescence signal and perform linear regression to determine the linearity of each DNA dye concentration for each acquisition setting **[1]**.

2.10.1. SCREEN: 67768_screenshot_1.mp4 00:12-00:15.

2.11. Choose the DNA dye concentration and acquisition settings that offer the best combination of linearity and dynamic range **[1]**.

2.11.1. SCREEN: 67768_screenshot_1.mp4 00:16-00:25.

3. Cell Plating, In-Well Drug Application, and Dead Cell Fluorescence Measurement Over Time in Drug-Treated Plates

3.1. After counting the cells, mix the counted cell suspension with the appropriate media volume using a serological pipette **[1]**. Transfer this suspension to a V-bottom reagent reservoir **[2]**.

3.1.1. Talent using serological pipette to mix cells with medium. NOTE: 3.1.1. and 3.1.2. are combined.

3.1.2. Talent transferring cell mixture into a V-bottom reservoir.

3.2. Using a multichannel pipette, add 90 microliters of the cell suspension to each well of the 96-well plates and mix the cell suspension regularly by repeated pipetting to maintain the desired cell concentration **[1]**.

3.2.1. Talent pipetting cell suspension into 96-well plate and mixing cell suspension by pipetting up and down.

3.3. Using a 10x concentration of the selected DNA dye in complete growth media, prepare a 10x concentration of each drug to be tested **[1]** and serially dilute using a multichannel pipette. Mix 15 times between each well **[2]**. NOTE: The VO for 3.3.2 has been deleted.

3.3.1. Talent preparing 10x concentration of the drug to be tested.

~~3.3.2. Talent creating the highest dose of a drug in the dilution plate.~~ Author's NOTE: 3.3.1 and 3.3.2 are saying the same thing. One shot was filmed for both of these steps.

3.3.3. Talent using a multichannel pipette to perform serial dilution and mixing by pipetting up and down in each well.

3.4. Now, add 10 microliters of the drug and DNA dye mixture to the plates containing cells **[1]**.

3.4.1. Talent pipetting drug + dye mixture into the cell-containing wells of the 96-well plate using a multichannel pipette.

3.5. Acquire fluorescence readings for all drug-treated plates every 3 to 4 hours after drug addition **[1]**.

3.5.1. SCREEN: 67768_screenshot_7.mp4 00:12-00:22, 00:40-00:45, 01:15-01:19.

3.6. At the final desired time point, acquire a fluorescence reading **[1]**. Immediately after that, add 10 microliters of 1.5 percent Triton-X solution to lyse the cells **[2]**.

3.6.1. SCREEN: 67768_screenshot_8.mp4 00:01-00:20, 00:40-00:45, 01:00-01:05.

3.6.2. Talent adding 10 microliters of Triton-X solution to each well.

3.7. Then, acquire fluorescence readings following cell permeabilization **[1]**.

3.7.1. SCREEN: 67768_screenshot_9.mp4 00:10-00:23, 00:40-00:45, 01:01-01:08. **TXT: The fluorescence value ∝ total cell count (live + dead) for each well; The post-permeabilization fluorescence value is the final population size for each well**

4. Calculation of the Lethal Fraction Kinetics

4.1. Calculate the average fluorescence values from the T0 *(T-zero)* control plate using the 50 percent trimmed mean **[1-TXT]**.

4.1.1. SCREEN: 67768_screenshot_2.mp4 00:00-00:07. **TXT: The average T0 fluorescence value is the initial population size for each well; The duration of the assay is from 0 h to the assay endpoint**

4.2. Using curve fitting and an exponential growth function, calculate the population growth kinetics for all wells **[1]**.

4.2.1. SCREEN: 67768_screenshot_2.mp4 00:10-00:13.

4.3. Based on the growth parameters, calculate the number of total cells at every measured time point in the assay for every well **[1]**. Now, subtract the dead cell measurement from the total cell counts to determine the number of live cells at each measured time point **[2]**.

4.3.1. SCREEN: 67768_screenshot_2.mp4 00:14-00:23.

4.3.2. SCREEN: 67768_screenshot_2.mp4 00:24-00:29.

4.4. Calculate the lethal fraction or LF at each time point by dividing the dead cell fluorescence signal by the total cell signal for each time point **[1]**.

4.4.1. SCREEN: 67768_screenshot_2.mp4 00:30-00:36.

4.5. Fit a Lag Exponential Death or LED *(L-E-D)* equation to the lethal fraction time course data **[1]**. For drug doses not causing significant lethality, use a linear model with zero slope. Determine significant levels of lethality based on the noise in the assay or the LF observed for undrugged conditions **[2]**. NOTE: The sentence numbers of the VO have been edited.

4.5.1. SCREEN: 67768_screenshot_2.mp4 00:37-00:58.

4.5.2. SCREEN: 67768_screenshot_2.mp4 01:00-01:03. NOTE: The timestamps for 4.5.3 is merged with 4.5.2.

~~4.5.3. SCREEN: To be provided by authors: Significant levels of lethality being determined based on the noise in the assay or the LF observed for undrugged conditions~~

4.6. From the LED equation, extract four parameters using non-linear regression: the initial lethal fraction or LFi *(L-F-I)*, lethal fraction plateau or LFp *(L-F-P)*, maximum death rate or DR *(D-R)*, and the death onset time or DO *(D-O)* **[1]**.

4.6.1. SCREEN: 67768_screenshot_2.mp4 01:04-01:13.

4.7. Compute the fractional viability or FV *(F-V)* at the assay endpoint for each drug at each

dose. Subtract the endpoint lethal fraction from 1 **[1]**, or divide the number of live cells by the total cells **[2]**. NOTE: The sentence numbers of the VO have been edited.

4.7.1. SCREEN: 67768_screenshot_2.mp4 01:14-01:17. NOTE: The timestamps for 4.7.2 is merged with 4.7.1.

~~4.7.2. SCREEN: To be provided by authors: The endpoint lethal fraction being subtracted from 1.~~

4.7.3. SCREEN: 67768_screenshot_2.mp4 01:18-01:28.

5. Calculation of GR Value and Drug-Induced Growth and Death Rates Using the GRADE Method

5.1. Determine the average number of live cells for each well at the start of the assay by calculating the difference between the post-permeabilization T0 reading and the T0 reading for each well. This value is referred to as x_0 *(Ex-zero)* **[1-TXT]**.

5.1.1. SCREEN: 67768_screenshot_2.mp4 01:29-01:35. **TXT: Determine the average number of live cells per well at the assay endpoint for both x_{ctrl} and each drug-treated condition or x(c)**

~~5.2. For the control wells or x_{ctrl} *(Ex-Control)*, determine the average number of live cells for each well at the assay endpoint **[1]**. Then, determine the average number of live cells at the assay endpoint for each drug-treated condition or x(c) *(X-C)* **[2]**.~~ NOTE: This step has been omitted. The VO for 5.2. has been added concisely as an onscreen text to 5.1.

~~5.2.1. SCREEN: To be provided by authors: The average number of live cells for each well at the assay endpoint being determined for the control wells.~~

~~5.2.2. SCREEN: To be provided by authors: The average number of live cells at the assay endpoint for each drug-treated condition being determined.~~

5.3. Now, for each drug-treated well, compute the normalized growth rate inhibition value or GR *(G-R)* using the given equation **[1]**. Perform curve fitting using a 4-parameter logistic regression **[2-TXT]**.

5.3.1. TEXT on PLAIN BACKGROUND:

$$\mathrm{GR(c)} = 2^{\log_2\left(\frac{x(c)}{x_0}\right)/\log_2\left(\frac{x_{ctrl}}{x_0}\right)} - 1$$

5.3.2. SCREEN: 67768_screenshot_2.mp4 01:35-01:51. **TXT: GR is on a scale from -1 to 1**

5.4. Next, determine the relationship between growth and death rates and the population size using a simulation based on a birth-death model **[1]**. Use the average number of live cells from the T0 control plate, the length of the assay, and user-defined ranges for plausible proliferation and death rates as inputs for this simulation **[2]**.

5.4.1. SCREEN: 67768_screenshot_2.mp4 01:52-02:00.

5.4.2. SCREEN: 67768_screenshot_2.mp4 02:00-02:09.

5.5. To determine plausible growth rates, use the untreated growth rate in cell population doublings per hour as the highest possible rate and zero as the lowest. Divide this range into 500 equally spaced segments **[1]**.

5.5.1. SCREEN: 67768_screenshot_2.mp4 02:10-02:27.

5.6. Determine the GR values for each simulated proliferation and death rate pair **[1]**. Then, determine the LF values **[2]**.

5.6.1. SCREEN: 67768_screenshot_2.mp4 02:28-02:32.

5.6.2. SCREEN: 67768_screenshot_2.mp4 02:33-02:44.

5.7. Compute the pairwise distance between each calculated GR and LF pair and each theoretical GR and LF pair in the look up table **[1]**. Identify the theoretical pair with the minimum distance to the experimentally observed GR and LF pair as the true drug-induced proliferation and death rates **[2]**.

5.7.1. SCREEN: 67768_screenshot_2.mp4 02:45-02:50.

5.7.2. SCREEN: 67768_screenshot_2.mp4 02:51-03:02.

Results

6. Representative Results

6.1. This figure illustrates how the FLICK *(Flick)* assay and GRADE *(Grade)* analysis quantify and distinguish between drug-induced growth inhibition and cell death in U2OS *(U-two-O-S)* cells **[1]**.

6.1.1. LAB MEDIA: Figure 2.

6.2. Belinostat at 1 micromolar showed a GR value of 0, indicating cytostasis **[1]**, but also caused approximately 50% cell death **[2]**.

6.2.1. LAB MEDIA: Figure 2A. *Video Editor: Highlight the "1 µM" point.*

6.2.2. LAB MEDIA: Figure 2B. *Video Editor: Highlight the 1 µM plot.*

6.3. GRADE analysis showed that 1 micromolar Belinostat caused both growth inhibition and cell death, with distinct contributions to overall population stasis **[1]**.

6.3.1. LAB MEDIA: Figure 2D, 2E.

6.4. The apoptotic inhibitor z-VAD (Z-Vad) reduced Belinostat-induced lethality by 18% **[1]**, indicating apoptosis involvement **[2]**.

6.4.1. LAB MEDIA: 2F. *Video Editor: Highlight the red line indicating the gap between the lavender and black curves and the equation ΔLF = 0.18.*

6.4.2. LAB MEDIA: 2F.

6.5. Conventional analysis showed all drugs reduced viability **[1]**, but GR analysis revealed only Belinostat and Camptothecin caused cell death at high doses **[2]**.

6.5.1. LAB MEDIA: 2G.

6.5.2. LAB MEDIA: 2H.

6.6. GRADE analysis showed Belinostat induced both growth inhibition and death at all doses **[1]**, Camptothecin was biphasic **[2]**, and Palbociclib caused growth arrest only **[3]**.

6.6.1. LAB MEDIA: Figure 2I. *Video Editor: Highlight the Belinostat bar graph.*

6.6.2. LAB MEDIA: Figure 2I. *Video Editor: Highlight the Camptothecin bar graph.*

6.6.3. LAB MEDIA: Figure 2I. *Video Editor: Highlight the Palbociclib bar graph.*

Pronunciation Guides:

1. Triton-X

Pronunciation link:
https://www.howtopronounce.com/triton-x
IPA: /ˈtraɪtɑn ɛks/
Phonetic Spelling: try-tahn eks

2. Morphologies

Pronunciation link:
https://www.merriam-webster.com/dictionary/morphology
IPA: /mɔːrˈfɑːlədʒiz/
Phonetic Spelling: mor-faa-luh-jeez

3. Microliter

Pronunciation link:
https://www.merriam-webster.com/dictionary/microliter
IPA: /ˈmaɪkrəˌliːtər/
Phonetic Spelling: my-kruh-lee-ter

4. Serological

Pronunciation link:
https://www.merriam-webster.com/dictionary/serological
IPA: /ˌsɪrəˈlɑːdʒɪkəl/
Phonetic Spelling: seer-uh-laa-juh-kl

5. Permeabilize

Pronunciation link:
https://www.howtopronounce.com/permeabilize
IPA: /ˈpɜːrmiəˌbɪlaɪz/
Phonetic Spelling: pur-mee-uh-bih-lyze

6. Fluorescence

Pronunciation link:
https://www.merriam-webster.com/dictionary/fluorescence
IPA: /flʊˈrɛsəns/
Phonetic Spelling: floo-reh-sens

7. Regression

Pronunciation link:
https://www.merriam-webster.com/dictionary/regression
IPA: /rɪˈgrɛʃən/
Phonetic Spelling: rih-greh-shuhn

8. Lethality

Pronunciation link:
https://www.merriam-webster.com/dictionary/lethality

IPA: /liˈθæləti/
Phonetic Spelling: lee-tha-luh-tee

9. Apoptotic

Pronunciation link:
https://www.howtopronounce.com/apoptotic
IPA: /ˌæpəpˈtɑːtɪk/
Phonetic Spelling: ap-uhp-taw-tik

10. Camptothecin

Pronunciation link:
https://www.howtopronounce.com/camptothecin
IPA: /ˌkæmptəˈθiːsɪn/
Phonetic Spelling: kamp-toh-thee-sin

11. Palbociclib

Pronunciation link:
https://www.howtopronounce.com/palbociclib
IPA: /pælˈbɒsɪklɪb/
Phonetic Spelling: pal-baa-sih-klib

12. Belinostat

Pronunciation link:
https://www.howtopronounce.com/belinostat
IPA: /bəˈlɪnəstæt/
Phonetic Spelling: buh-lih-nuh-stat