A high-throughput platform for assessing synergy of drug combinations

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As novel therapies continue to be discovered and diseases continue to grow resistant to current treatments, the need has emerged to rapidly assess drug combinations as viable treatments. Here, we describe a simple, robust, and high-throughput system which can be used to test combinations of agents that may have a mechanistic basis for synergy, such as proteasome and histone deacetylase inhibitors in multiple myeloma, or combinations of new drugs that may not have obvious mechanisms of action, but may yield more efficacious patient outcomes when synergistically used in concert. Currently, drug combination studies can be tedious, labor-intensive, involve advanced experimental setups, and require complicated mathematical analysis and data processing. Using a single 384-well assay plate and one-step analysis package in R, our process streamlines the analysis of drug combination studies, generates intuitive and revealing graphical representations, and rapidly quantifies synergy of 25 different dose combinations in a single experiment.

INTRODUCTION

Drug combinations are some of the most widely and historically used therapies in devastating diseases such as cancer and the human immunodeficiency virus (HIV) (DeVita and Schein, 1973; Pakker et al., 1998). Often, combining two drugs with similar effects results in enhanced potency, but to assess whether the combined affect is greater than the expected sum of the individual potencies requires an assessment of synergy. True synergism in drug combinations allows for not only reduced dosing of both drugs involved, but can also reduce adverse effects and provide novel therapeutic avenues for the most mechanistically confounding diseases(Chou, 2010). Commonly in cancer, selection of combination strategies is stochastic, based on drug availability, and limited to established dosing principles in patients from single-agent therapeutic index studies(Greco et al., 1995).

With the recent development of a host of promising drug candidates emerging from investigator-initiated research labs, a high-throughput but facile method for assessing drug combinations is needed. For these research groups, combination studies are often considered out-of-reach due to limited quantities of new compounds, and researchers often feel compelled to hold-off on the assessment of synergy until the most promising candidate is identified and rigorously confirmed. While many new computational methods are being developed to predict likely drug combination strategies(Crystal et al., 2014; Folger et al., 2011; Soldi et al., 2013), current methods to assess these many possible hypotheses are laborious. Further, for rare diseases which are often studied in academic institutions rather than major pharmaceutical companies(Frye et al., 2011), a high-throughput method for assessing drug synergy is particularly valuable. For these diseases especially, investigator-initiated clinical trials get few "shots-on-goal," and drug synergy is not a priority, even though drug combinations are often the most valuable and effective therapeutic strategies(Frye et al., 2011). Our method provides an avenue to rapidly assess promising combination therapeutics, doses, and classes of drug. This facile approach is ideal for rapidly assessing either a single drug combination across a host of cell lines, the combination of a single molecule with a variety of compounds or mechanisms, and may help improve the utility and translation of new combination therapies from the benchtop to the clinic.

Combination studies are often tedious and involve several experimental setups, cumbersome data entry into combination analysis software, and large time investments into deciphering the resulting data. Our high-throughput design streamlines this process by using a single compound plate format

Box 1 | Considerations for choosing dosing concentrations

To appropriately calculate synergy with the Chou-Talalay method, generating a reliable median-effect equation from the individual dose responses is the most critical component. While our method includes an internal control, it is important to pick 5 doses that adequately span each Drug's IC50, even if the effects are minimal. For example, if a drug reaches maximum toxicity at 100nM, and has no effect at 5 nM, ideal dose treatments would be 100 nM, 50 nM, 25 nM, 12.5 nM, and 6.25 nM. Since not all molecules are active in a broad spectrum of 2-log doses, our protocol may be easily adjusted to smaller ranges of drug combinations through the use of a normalized isobolgram. This protocol is not limited to 2-fold dilution series if the maximum and minimum dose choices are not suitable (i.e 5 Doses 500 nM, 400 nM, 300 nM, 200 nM, 100 nM), however if one is performing the protocol with manual pipetting rather than pinning, the dilution protocol (Option 2, Steps 5-14) must be adjusted accordingly.

Further, given that certain drugs may have varying pharmacokinetics and serum stability, it is recommended that the protocol be adjusted according to the best practices of the molecules being tested. For each molecule being tested, we recommend confirming plasma stability prior to performing the assay (Box 2). In certain cases, especially if a given cell line requires greater than 2 days of treatment to observe measureable affects, it is highly recommended to replenish the media on the assay plate after 48 hours with fresh drug, as in many cases this can drastically improve the dynamic range of synergy assessment and yield a more accurate quantification of synergy. To replenish media, we recommend completely inverting the assay plate into a sterile waste bin inside the hood, with one rapid flick to remove all spent media simultaneously.

that can be used multiple times, one assay plate per combination, and an R-package that takes raw data in simple text format and generates useful and revealing graphic renderings of the data. The method can be easily extended to even the most basic laboratory set-ups, including those without access to a pinning robot or pinning apparatus. Further, our platform provides a facile method for assessing drug-drug combination studies even when drug amounts are limited, as it requires only a few microliters of compound per assay.

The Chou-Talalay method for drug synergy assessment is a powerful tool which provides a rigorous, quantitative analysis of drug combinations(Chou, 2010; Chou and Talalay, 1984). Several methods have been proposed to assess synergy, with rather contradictory definitions of synergy(Greco et al., 1995). Many of these methods require industrialized, expensive equipment, large quantities of compound, and often sacrifice the mathematical rigor of the Chou-Talalay method. We therefore sought to develop a facile platform for assessing a broad range of dose combinations, which is applicable to chemical libraries both large and small, with a universally accessible analysis, which can be performed with limited quantities of compound. By combining elements of the Scatchard, Michaelis-Menten, Hill, and Henderson-Hasslebalch equations though the law of mass-action, the Chou-Talalay method provides a mechanism-independent approach to generate a quantifiable assessment of drug synergy, which is known as the Combination Index (CI)(Chou, 2006; 2010). To determine a CI, our package first generates median-effect equations of the two-respective molecules being tested internally in each experiment to account for experiment-to-experiment, cell line-to-cell line, or drug-to-drug variations. Using the median-effect equation, the package calculates a median-effect dose, and $(D_x)_{A,B}$ values, which correspond for the respective doses of drugs A and B which correlate with a given percentage of cells affected by the individual treatments(Chou, 2010). Normalized index values (I_A, I_B), and the combination index are a ratio between the treatment dose, and the D_x for a given fraction of affected cells(Chou, 2010). The package generates an individual CI for 25 individual dose combinations per experiment, and an overall average CI for all the combinations tested. To determine synergy, combination indices less than 0.9 are considered synergistic, while CI above 1.1 are antagonistic and values ranging from 0.9 to 1.1 are additive(Chou, 2010).

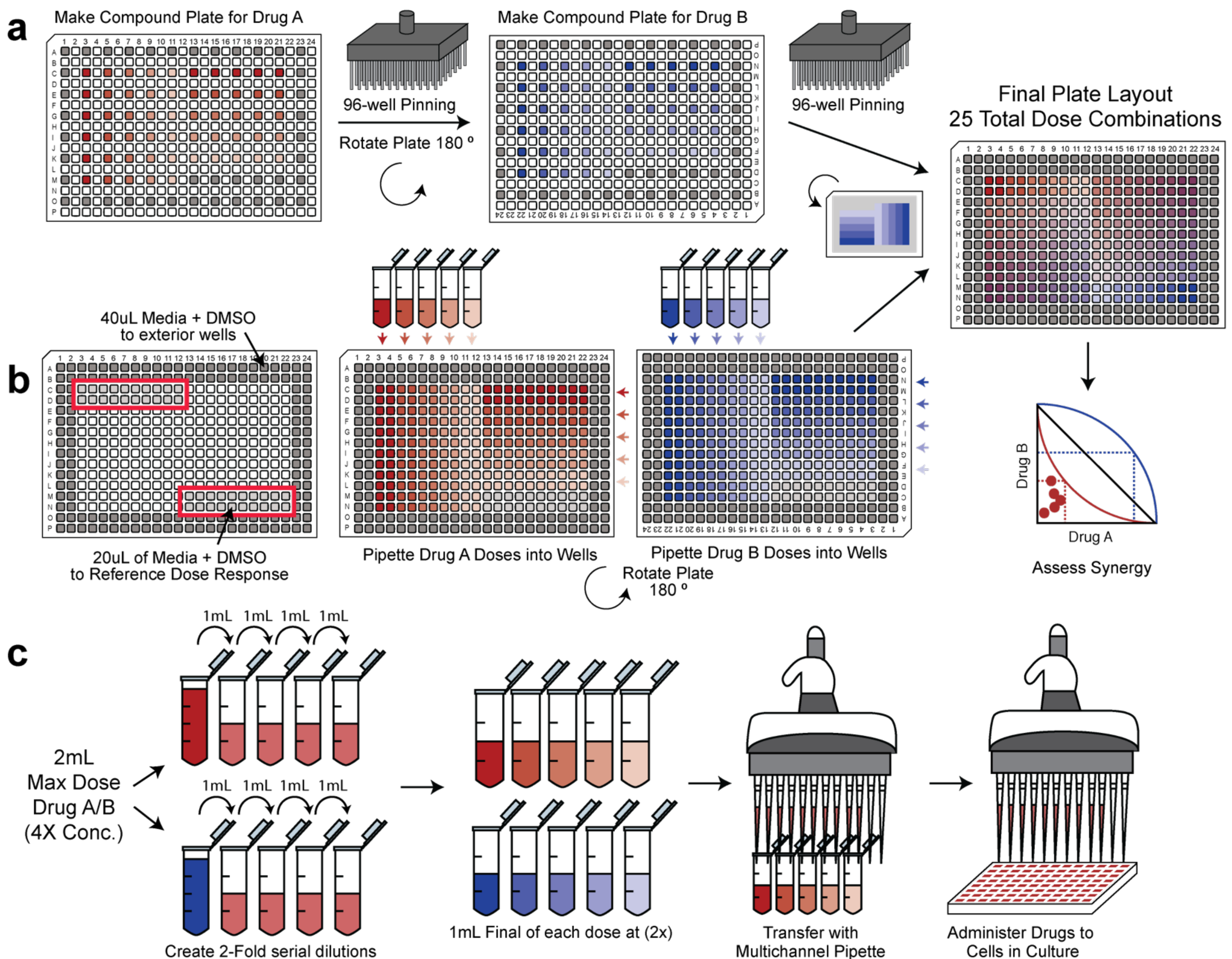

Figure 1 | Schematic overview of synergy high-throughput synergy protocol

PROCEDURE

Cell Culture (2-5d)

1. *Seed Cells.* In a black, clear-bottomed 384-well dish, seed cell line of interest in appropriate medium. **For suspension cell lines:** seed at 2000 cells/well in 40 µL of media/well. **For adherent cell lines:** seed at 500 cells/well in media in 40 µL media/well.
2. Grow cells overnight in appropriate medium under desired conditions.

(Option 1) Prepare Drug combinations using 96-well pins ● TIMING ~2h

3. Create stock compound plates for Drug A and Drug B using format shown in Figure 1A by filling each control well (gray) with 11 µL of DMSO and by filling each dilution series with 10 µL of 400X of Drug A or Drug B in DMSO for each respective dose. A total of 5 different doses will be tested for each of the two drugs in the assay.

PAUSE POINT: Compound plate may be prepared in advance and stored at 4°C if compounds tolerate freeze/thaw

4. The next day, transfer 100 nL of compound from the compound plate to cell culture plate with a 96-well pinning apparatus. Transfer the compound in a quad format, such that 1 well from the compound plate is delivered to the assay plate.
5. Incubate cells at desired conditions for 48h
6. *[Optional]* Refresh media after 48 hours (repeating steps 6-7) and incubate for additional 48 hours
7. Proceed to data collection.

(Option 2) Prepare Drug combinations using manual pipetting ● TIMING ~2-4h

3. Add 2 mL of fresh media to a 2mL Ependorff tube. To the 2 mL of media, add Drug A to media such that the concentration is 4x the highest treatment dose. For example, if the range of 5 doses to be tested is from 20 µM – 1.25 µM, the concentration should be 2 mL of 80 µM in fresh media.
4. Create 4 serial dilutions of Drug A (5 doses total) by transferring 1mL of the highest dose into 1mL of fresh media containing 1:1 amounts of DMSO for every µL of compound added to the first tube. It is critical that the total amount of DMSO in each tube is equivalent, even following serial dilutions, to

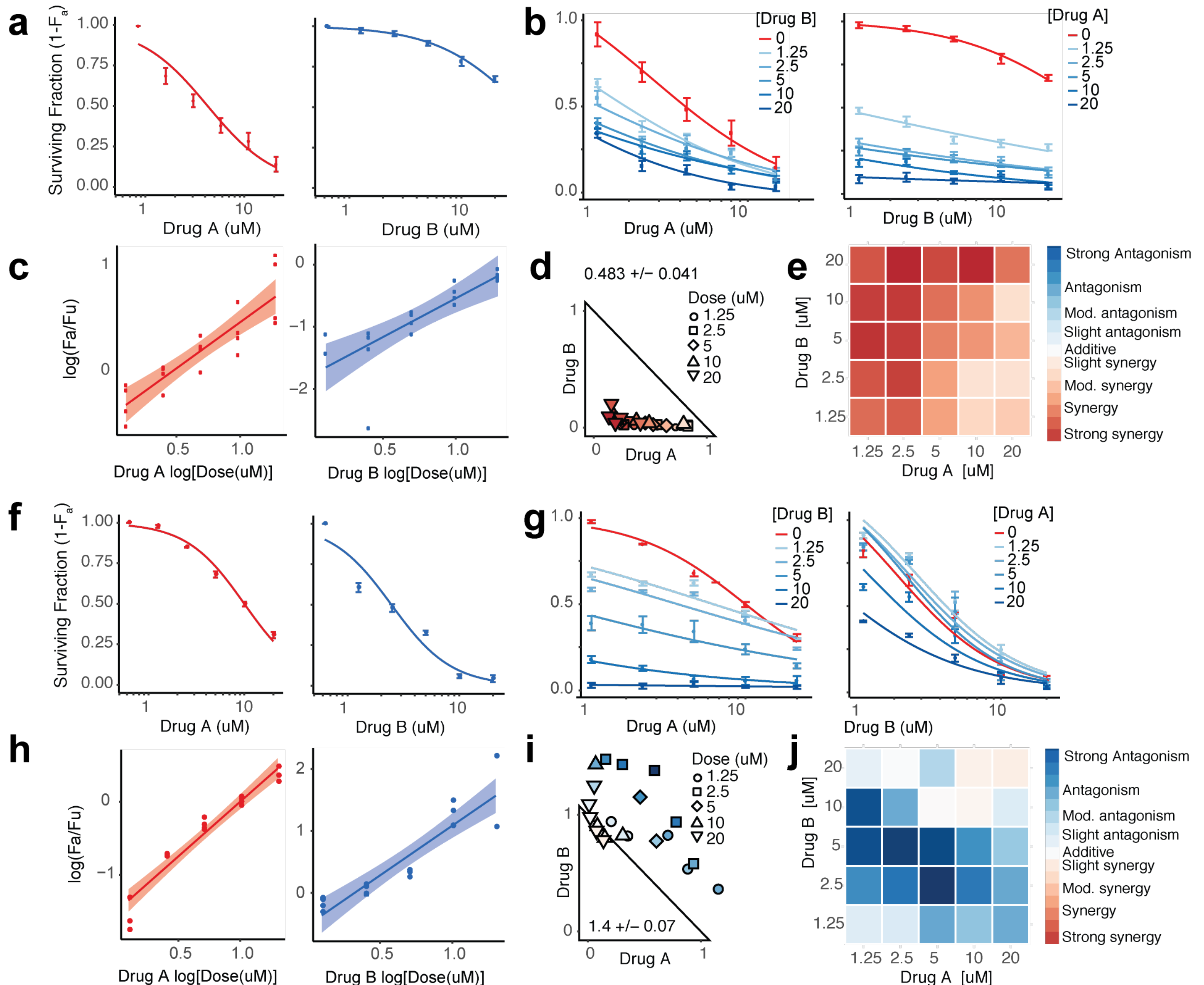

Figure 2 | Representative Data generated by SynergyCalc package for two drugs demonstrating either synergism or antagonism.

ensure that effects of DMSO have no bearing on the calculations with the lowest doses tested (Box 1B). You now should have 5 tubes containing 1mL each of 2-fold dilutions of Drug A.

5. Repeat steps 3-4 for Drug B.
6. To two rows of exterior wells, add **40µL** fresh media containing DMSO, as described in Figure 1C. *(Rows: A-B[1-24], O-P[1-24], Cols: 1-2[A-P], 23-24[A-P]).* To Internal dose response control wells, add **20µL** fresh media containing DMSO, as described in Figure 1C. *(Wells: C-D[3-12], M-N[13-22])*
7. Utilizing a multi-channel pipette, add **20µL** of each dose of Drug A left side of plate (2 replicates per dose per row), as described in Figure 1C. *(Rows **C-N**, Columns 3-12. Beginning at Row C and working to N).*
8. Following Figure 1C, add **20µL** of each dose of Drug A to right side of plate (2 replicates per dose per column), as described in Figure 1C. *(Rows **C-L**, Columns 13-22. Beginning at Column 13 and working to 22).*
9. Rotate entire assay plate 180° and repeat steps 11-12 with Drug B, will all rows and columns inverted, as described in Figure 1C.
10. Incubate cells at desired conditions for 48h
    [Optional] Refresh media after 48 hours (repeating steps 3-10) and incubate for 48 additional hours. **Note:** Each well should now have a total of 80 µL (40 from seeding previous day, 20 Drug A, 20 Drug B
11. Proceed to Data Collection

Data Collection ● TIMING ~1-4h

12. For cell viability, the CellTitre Blue (alternatively Cell Titre Glo) assay is recommended. **NOTE:** Alternative synergy read-outs (Alpha-LISA assays, reporter assays, etc. should be performed according to manufacturer's instructions or established protocols).
13. To each well, add 5 µL of CellTiterBlue® and Incubate assay plate for 4h at 37C and read plate at 560/590 em/ex with a 384-well luminometer.

Data Processing ● TIMING < 1h

14. Save data file as a tab-delimited text file, with no headers or column names (16 x 24 matrix).
15. If not previously installed, install R packages `"RColorBrewer"`,`"dplyr"`, `"ggplot2"`, `"reshape"`, `"devtools"`, and `"scales"`, utilizing the function `install.packages()` [i.e. `install.packages(RColorBrewer")`]. Load devtools package (i.e. `library(devtools)`) and install synergy analysis package using `install.github("EChormatin/SynergyCalc")`.
16. For one-step analysis, assign the following parameters into the `synergycalc()` function and execute: 1) name of DrugA, name of DrugB, cell line, 5 doses of Drug A (from high to low), 5 doses of Drug B (from high to low), location of data file(s), location where figures/analyzed data should be exported.
    Example :

```
synergycalc(Drug1 = "My Drug A",
            Drug2 = "My Drug B",
            cell_line = "HEK293T",
            Dose1_list = c(20, 10, 5, 2.5, 1.25),
            Dose2_list = c(40, 20, 10, 5, 2.5),
            filename = "Desktop/Example Synergy data.txt",
            filename2 = "Desktop/Example Synergy data Rep.txt", #Up to 6 replicate files
            file_dest = "Desktop/Example Output Folder/")
```

Box 2 | Assessing *in vitro* compound stability

If the synergy assay is to be performed for time points longer than 48 hours, we highly recommend confirming the stability of the compounds, especially if testing new small molecules or peptides. If the molecules undergo significant degradation at 48h, it is highly recommended that the media be replenished halfway through the assay, given that compounds are subjected to degradation/modification (particularly hydrolysis) which can have adverse effects on synergy analysis.
[Note]: if the amount of compound is extremely limited, this analysis may not be possible.

For many small molecules:

1. Prepare two solutions of test compound (100uM and 10uM) in 100 µL of cell culture medium containing 10% FBS.
2. Remove 25µL aliquots at pre-defined time points (t = 0, t = 180 min, t = 6h, t = overnight)
3. To each sample, add an equal volume of internal standard (IS) spike-in solution [100 µM procaine (positive control), 100 µM procainamide (negative control) in 95% water, 4.9% acetonitrile, and 0.1% DMSO], followed by an equal volume of blank spiking solution [95% water, 4.9% acetonitrile, and 0.1% DMSO].
4. To generate standard curves, to 50µL cell culture media, add an equal volume of IS spiking solution, followed by an equal volume of test compound spiking solution (10 ng/mL to 10 µg/mL in 95% water, 4.9% acetonitrile, and 0.1% DMSO).
5. Increase the volumes of all samples to 250 µL by the addition of water and mix well. Then, add 600µL of ethyl acetate to the samples, shake thoroughly and vortex. Separate the phases by brief centrifugation and then withdraw 500 µL of ethyl acetate and place into a separate microcentrifuge tube. Add 300 µL of new ethyl acetate, and repeat the extraction and combine the extracts.
6. To reconstitute the compounds, evaporate the ethyl acetate, add 20 µL of methanol to each tube, and vortex thoroughly. Briefly centrifuge and add 80 µL of water with 0.1% formic acid and vortex thoroughly again.
7. Finally, withdraw the liquids, pass the solution through 0.45 µm centrifugal filter unit and analyze by LC-MS (ESI-QTOF).

17. For step-by-step analysis, execute the following functions with the same parameters as step 24:
    a. Export viability data : `write.synergy.rawdata()`
    b. Individual dose responses : `synergycalc.indv.dose()`
    c. Median-effect plots : `synergycalc.median.effect()`
    d. Shifting IC50 plots : `synergycalc.ShiftingIC50s()`
    e. Fraction-affected grid : `synergycalc.FAplot()`
    f. Normalized Isobologram : `synergycalc.isobologram()`
    g. Combination index grid : `synergycalc.CI()`
    h. Average combination index : `synergycalc.CI.mean()`
    i. Export synergy data : `write.synergy()`

MATERIALS

Reagents

- DMEM-high glucose medium (Invitrogen, cat. no. 11960-044)
- l-Glutamine (Invitrogen, cat. no. 25031-081)
- Penicillin-streptomycin (Invitrogen, cat. no. 15070-063)
- FBS, qualified and heat inactivated (Life Technologies, cat. no. 10438-034)
- Trypsin-EDTA solution, 0.05% (wt/vol) (Invitrogen, cat. no. 25300-062)
- CellTiter-Blue Cell Viability Assay (Promega, cat. no. G8081)
- Dimethyl sulfoxide (Sigma Aldrich, cat. no. D2650)

EQUIPMENT

- Filtered sterile pipette tips (Corning)
- 125uL Matrix Tips (Thermo Scientific, cat. no. 7322)
- Pipette Reservoir (Fisher Scientific, cat. no. 13-681-501)
- 384-well clear bottom plate (E&K Scientific, cat. no. EK-30091)
- Standard microcentrifuge tubes, 2 ml (Corning, Product #MCT-200-NC)
- Multi-channel pipette (Thermo Scientific, cat. no. EW-94999-47)
- 384-well fluorescent plate reader (Perkin Elmer, cat. no. HH34000000)
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Box 3 | Selecting Drug Combinations

When identifying possible targets for synergy assessment, it is important to begin with compounds that target different mechanisms of action, including different targets, targets within pathways, or even inhibitors of the same target that act through independent mechanisms(Jia et al., 2009). Cancer therapy combinations are uniquely sensitive to drug combinations which act through different pathways and mechanisms ranging from inhibitors of DNA synthesis and repair (Tanaka et al., 2005, Chory et al (submitted)), to epigenetic regulation(Mazur et al., 2015; Shu et al., 2016) or inhibition of signaling cascades (Jia et al., 2009). In combination treatments for diseases such as HIV which are highly susceptible to multi-drug resistance through evolution, compounds inhibiting the same target through different mechanism of action, such as in the case of nucleoside (AZT) and non-nucleoside HIV-1 RT inhibitors, may be desired (Fattorusso et al., 2005). It is also important to consider that given that the Chou-Talalay method is a mechanism-independent assessment of synergy(Chou, 2010), that the results of this analysis are not intended to clarify a mechanism of compounds with unknown mode-of-actions. Synergy may be observed between compounds with mechanisms as similar as two different cell cycle checkpoint-inhibitors, or as varied as combining a regulator DNA synthesis, with an inhibitor of drug import or export. Alternatively, drugs that target clearly independent pathways, do not necessarily correlate with increased synergy. For this reason, a high-throughput method to rapidly assess novel, unexpected, or even predicted drug in will facilitate the discovery of novel combination therapies.

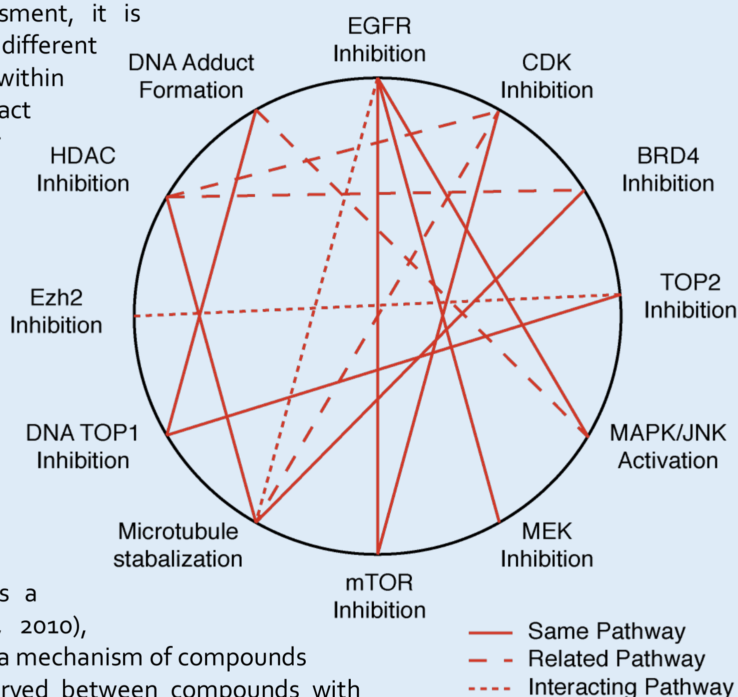

FIGURE LEGENDS

Figure 1 | Schematic overview of high-throughput synergy protocol **(a)** Schematic of compound plate layout and experimental method for assessing the synergy between Drug A (red) and Drug B (blue) using a 96-well pinning apparatus or robot. Decreasing color saturation corresponds to decreasing drug combinations. White corresponds to empty wells for pinning, grey corresponds to wells filled with DMSO. **(b)** Schematic of compound plate layout workflow for assessing the synergy between Drug A (red) and Drug B (blue), with hand or repeat-pipette distribution of media containing each compound. Media containing DMSO is added to exterior wells and control wells first, followed by media containing Drug A (Red), and Drug B (Blue), with the final plate layout shown (far right, a-b). (c) Schematic **(c)** Schematic for creating appropriate volumes of serial dilutions for Drug A (red) and Drug B (blue) when transferring media by hand, or with a repeat pipettor.

Figure 2 | Representative Data generated by SynergyCalc package for two drugs demonstrating either synergism or antagonism. **(a)** Representative individual dose response curves, **(b)** shifting dose response curves, **(c)** median effect plots, **(d)** normalized isobologram, and **(e)** combination index plots, for Drug A (red) and Drug B (blue) that when combined, demonstrate synergy. **(f)** Representative individual dose response curves, **(g)** shifting dose response curves, **(h)** median effect plots, **(i)** normalized isobologram, and **(j)** combination index plots, for Drug A (red) and Drug B (blue) that when combined, demonstrate antagonism.

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