Video Article

High-throughput Identification of Synergistic Drug Combinations by the Overlap^2 Method

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URL: https://www.jove.com/video/57241
DOI: doi:10.3791/57241

Keywords: Genetics, Issue 135, Antibiotic resistance, antimicrobial resistance, drug combinations, drug synergy, high-throughput screen, Overlap^2 Method

Date Published: 5/21/2018

Citation: Wambaugh, M.A., Brown, J.C. High-throughput Identification of Synergistic Drug Combinations by the Overlap^2 Method. *J. Vis. Exp.* (135), e57241, doi:10.3791/57241 (2018).

Abstract

Although antimicrobial drugs have dramatically increased the lifespan and quality of life in the 20th century, antimicrobial resistance threatens our entire society's ability to treat systemic infections. In the United States alone, antibiotic-resistant infections kill approximately 23,000 people a year and cost around 20 billion USD in additional healthcare. One approach to combat antimicrobial resistance is combination therapy, which is particularly useful in the critical early stage of infection, before the infecting organism and its drug resistance profile have been identified. Many antimicrobial treatments use combination therapies. However, most of these combinations are additive, meaning that the combined efficacy is the same as the sum of the individual antibiotic efficacy. Some combination therapies are synergistic: the combined efficacy is much greater than additive. Synergistic combinations are particularly useful because they can inhibit the growth of antimicrobial drug resistant strains. However, these combinations are rare and difficult to identify. This is due to the sheer number of molecules needed to be tested in a pairwise manner: a library of 1,000 molecules has 1 million potential combinations. Thus, efforts have been made to predict molecules for synergy. This article describes our high-throughput method for predicting synergistic small molecule pairs known as the Overlap^2 Method (O2M). O2M uses patterns from chemical-genetic datasets to identify mutants that are hypersensitive to each molecule in a synergistic pair but not to other molecules. The Brown lab exploits this growth difference by performing a high-throughput screen for molecules that inhibit the growth of mutant but not wild-type cells. The lab's work previously identified molecules that synergize with the antibiotic trimethoprim and the antifungal drug fluconazole using this strategy. Here, the authors present a method to screen for novel synergistic combinations, which can be altered for multiple microorganisms.

Video Link

The video component of this article can be found at https://www.jove.com/video/57241/

Introduction

Antibiotic-resistant bacteria cause more than 2 million infections and 23,000 deaths annually in the United States according to the CDC[1]. New treatments are needed to overcome these infections. Strategies to identify these new treatments include the development of new antimicrobial drugs or the repurposing of small molecules approved for other conditions to treat microbial infections[2,3,4]. However, new drug discovery is very costly and time-consuming. Repurposing drugs may not identify novel drugs or drug targets[5,6]. Our lab focuses on a third strategy known as synergistic combination therapies. Synergistic combinations occur when two small molecules together have an efficacy greater than the additive effect of their individual efficacies[7]. Additionally, synergistic combinations can be effective against a pathogen resistant to one of the small molecules in the pair in addition to having less unwanted off-target effects, rendering them great potential[8,9,10].

Synergistic pairs are rare, occurring in approximately 4-10% of drug combinations[11,12,13]. Thus, traditional techniques such as pairwise screens are challenging and time-consuming, with thousands of potential combinations from a small library of a hundred molecules. Moreover, synergistic interactions usually cannot be predicted from the activity of the compounds[14]. However, the authors developed a high-throughput approach to screen for synergistic pairs, called the Overlap^2 Method (O2M)[12]. This method, described here, allows for faster, more efficient identification of these synergistic pairs. O2M requires the use of a known synergistic pair and a chemical-genetics dataset. Chemical-genetics datasets are generated when a library of knockout mutants is grown in the presence of many different small molecules. If one molecule in a known synergistic pair induces the same phenotype from a particular knockout mutant as the second synergistic molecule, any other small molecule that elicits the phenotype from that same mutant should also synergize with each member of the known synergistic pair. This rationale has been used in the Brown lab to identify synergistic antibiotic pairs active against *Escherichia coli (E. coli)* and synergistic antifungal drug pairs active against the pathogenic fungus *Cryptococcus neoformans* (*C. neoformans*)[11,12]. O2M is not only adaptable for various pathogens, but allows for the screening of large libraries of molecules to identify synergistic pairs easily and rapidly. Screening with the genetic mutant identified by O2M allows us to validate only those small molecules predicted for synergy. Thus, testing a 2,000-molecule library pairwise would take months, whereas if there were only 20 molecules in that library predicted to synergize, testing for synergy now takes a matter of days. O2M does not require programming skills, and the required equipment is available in most labs or core facilities. In addition to researchers interested in drug combinations, O2M analysis is of interest to anyone who has completed a drug screen and wants to expand their hits by identifying important drug-drug interactions.

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Below is the protocol for identifying synergistic small molecules in bacteria, as well as validating the predicted synergistic interactions in well-known assays[15,16].

Protocol

1. Identifying Synergy Prediction Mutants from Chemical-genetics Dataset by the Overlap^2 Method (O2M)

NOTE: This is the method for identifying synergy prediction mutants using the published dataset from Nichols *et al*.[17] in *E. coli*. However, this can be done on any chemical-genetics dataset and microorganism. These data sets contain a library of knockout mutants grown in the presence of more than 100 small molecules, giving a quantitative growth score for each mutant in each small molecule. One synergistic pair must be known, and there should be growth scores for both small molecules included in the dataset.

1. **Calculate the mean growth score and standard deviation for all mutants grown in the presence of a single concentration of each small molecule.**
   NOTE: This can be done using any spreadsheet software. The Brown lab uses Excel and the functions =AVERAGE(B3:B3981) and =STDEV(B3:B3981) for the calculations of each column of small molecule.
   1. Calculate the means and standard deviations from each column of small molecule, though this orientation may differ if using a different dataset.
2. Calculate Z scores for each concentration of small molecule using the same dataset from Nichols *et al*.[17] in a spreadsheet program. For this, negative z scores will be Z(2.5) = mean -2.5*standard deviation, and positive z scores will be Z(2.5) = mean + 2.5*standard deviation.
3. Determine which gene mutants contain significant Z scores in the small molecules that make up the known synergistic pair, being sure to look at all concentrations of those molecules. If the growth score of a mutant is less than the negative Z (2.5) score or is greater than the positive Z (2.5) score for that small molecule, it is considered significant.
4. Determine if any of the significant gene mutants belong to an operon.
   NOTE: For *E. coli*, the authors recommend checking "Ecoli wiki" for information regarding if genes are transcribed as part of a polycistronic RNA.
5. Identify gene/operon mutants that are common to the majority of the small molecules of interest at different concentrations (*e.g.*, when looking for molecules that synergize with the antibiotic trimethoprim, identify mutants that show a significant Z score when grown in the presence of trimethoprim and its known synergistic partners such as sulfamethizole or sulfamethoxazole). These are putative synergy prediction mutants.

2. Predicting Synergizers within the Chemical-genetics Dataset by O2M

1. Returning to the dataset[17], identify each concentration of small molecule that elicits a significant growth score from the synergy prediction mutant. This is done by looking at the Z scores calculated for each concentration of small molecules. Again, a significant growth score is less than the negative Z score or greater than the positive Z score for that concentration of small molecule.
   NOTE: If a molecule appears at even one of its concentrations, the molecule is considered a predicted synergizer. Any molecule that does not elicit a significant growth score is a predicted non-synergizer.

3. Validation of Predicted Synergistic Interactions

1. **Finding minimal inhibitory concentration (MIC) of predicted synergizers.**
   1. Grow an overnight culture of *E. coli* in M9 minimal media at 37 °C.
      NOTE: Any overnight culture can be grown in appropriately defined medium for the organism of interest. The Brown lab does not recommend rich media like LB or YPD. M9 minimal media consists of 10.5 g/L M9 broth, 0.2% casamino acids, 0.1 M $CaCl_2$, 0.4% glucose, 1 M $MgSO_4$, 0.25% nicotinic acid, and 0.33% thiamine in H_2O.
   2. If no MIC data for pathogen of interest is found in the literature upon receiving the small molecules, dissolve in dimethyl sulfoxide (DMSO) at a high concentration (>10 mg/mL).
   3. Using a 96-well plate, add 100 µL of M9 media to each well. Add an extra 100 µL of M9 media in column 1 so it contains a total of 200 µL.
   4. Add an arbitrary amount (~10 µL) of concentrated small molecule solution in column 1. One small molecule per well of column 1 (8 small molecules per plate).
      NOTE: These are the small molecules of interest, that are predicted to synergize. The Brown lab generally adds 10 µL of highly concentrated small molecule solution, which is below the amount of 100% DMSO that can inhibit *E. coli*.
   5. Once the small molecules have been added, dilute the small molecules across the x-axis of the plate. Take 100 µL of solution from column 1, place in column 2 and mix. Take 100 µL from column 2, place in column 3 and mix. Continue this process until 100 µL are placed in column 11. Mix column 11, then take 100 µL from that column and discard. Leave column 12 with just media to normalize the data.
   6. Inoculate the plate. Obtain the optical density (OD_{600}) from an overnight culture. Prepare a solution with the culture and M9 media to an OD_{600} of 0.002 (or appropriate concentration that represents 500 cells/µL for the desired organism of interest). Pipette 2 µL of culture solution into each well of the plate so there are 1,000 cells per well.
   7. Gently shake the plate and obtain the OD_{600} for the 0 h reading. Let the plate grow at 37 °C for 24 h. Obtain the OD_{600} for the 24 h reading.
      NOTE: Use appropriate growing conditions for other organisms of interest.
   8. Calculate the net growth for each well using a spreadsheet program.

9. Average the net growth of column 12 to obtain the average no drug growth. Normalize the other wells to the average of no drug growth using a spreadsheet software. Look for any well with less than 10% growth to identify the MIC90. Any well with less than 50% growth indicates the MIC50. Calculate the concentration of small molecule in those wells.

2. **Checkerboard Assays for synergistic interactions**
   1. Grow an overnight culture of *E. coli* in M9 minimal media as before.
      NOTE: Use appropriate growing conditions for other organisms.
   2. Add 100 µL of M9 media to each well of a 96 well plate and an extra 100 µL in column 1.
   3. Add the test small molecule (at ~8x MIC) to each well in column 1 and add double the concentration (~16x MIC) to well A1 (one small molecule tested per plate).
      NOTE: This amount is determined from the MIC test completed prior and differs for each potential synergizer.
   4. Create a gradient dilution on the x-axis by taking 100 µL from column 1 and transferring to column 2. Continue this process until 100 µL is added to column 11. Mix, then take 100 µL from column 11 and dispose. Do not add any drug to column 12 (**Figure 1A**).
   5. Set up the second drug gradient across the y-axis for the input drug. Add 100 µL of media containing 2x MIC of the input molecule in row A. Mix and dilute as before, taking 100 µL from row A and placing in B. Continue until 100 µL is placed in row G. Mix, take 100 µL and dispose, ensuring not to add any input drug in row H. This allows row H and column 12 to contain individual MICs of the drug pair tested (**Figure 1B**).
   6. Inoculate the plate. Add 2 µL of a *E. coli* culture of OD_{600} 0.002 to each well (1,000 cells per well).
   7. Measure the OD_{600} of each well in the plate for the 0 h reading.
   8. Incubate the plate for 24 h at 37 °C.
   9. Measure the OD_{600} of each well at 24 h.

3. **Calculating the fractional inhibitory concentration index (FICI) from Checkerboards**
   1. Calculate the net growth for each well of the plate by subtracting the OD_{600} at 0 h from the OD_{600} at 24 h in a spreadsheet program.
   2. Normalize the growth in the software by dividing each well by well H12, which contains no small molecule.
   3. Find the MIC90 of the input molecule in column 12 and the MIC90 of the potential synergist in row H.
   4. Look for all wells that inhibit 90% of growth.
   5. Calculate the FICI90 for the well that is either the lowest below (synergistic) or highest above (antagonistic) both MIC90 values, using the equation:
      $$FIC\ index = FICx + FICy = \frac{x}{MICx} + \frac{y}{MICy}$$
   6. Consider any FICI ≤ 0.5 as synergistic and FICI ≥ 4 as antagonistic (**Figure 2**).
      NOTE: This can also be done with MIC50 (50% inhibition of growth) to get an FICI50 based on those MIC values.

4. **Bliss Independence Assay**
   NOTE: Bliss Independence is run with any potential synergist that did not have an MIC on its own.
   1. Grow an overnight culture of *E. coli* in M9 media at 37 °C, as before.
      NOTE: Again, appropriate growing conditions for other organisms of interest can be used in this step.
   2. Prepare 96-well plates for testing the potential synergistic molecule, the input molecule, the combination, and a non-treated control.
   3. Create the potential synergist plate by adding 50 µL of M9 media to each well. Add 50 µL of media containing a set concentration (10 µM or 100 µM) of the potential synergist to each well of a row. Test 8 molecules per plate.
   4. Create the input molecule plate by adding 50 µL of media to each well. Add 50 µL containing 4x MIC of the input drug to each well in column 1. Create a gradient dilution along the x-axis similar to MIC plates, taking 50 µL from column 1, dispersing and mixing in column 2. Continue this process until column 12, disposing 50 µL from column 12. Add an additional 50 µL of media to each well so the end total for each well is 100 µL.
   5. Creating the combination plate by using a 96 well plate, add 50 µL of media to each well. Add 50 µL containing 4x MIC of the input drug to each well in column 1. Dilute along the x-axis similar to MIC plates, taking 50 µL from column 1, dispersing and mixing in column 2. Continue this the whole way to column 12, disposing 50 µL from column 12. Add 50 µL of media containing the potential synergist at 10 µM or 100 µM to each well of a row. Again, there should be one concentration or drug per row.
   6. Create the non-treated control by adding 100 µL of M9 media to all wells of the plate.
   7. Inoculate all plates by adding 2 µL of bacterial culture with an OD_{600} of 0.002 or 1,000 cells to each well as before.
   8. Measure the OD_{600} of each well at 0 and 24 h.

5. **Calculating the Bliss Independence Score**
   1. Calculate the net growth for each well of the plate by subtracting the OD_{600} at 0 h from the growth at 24 h using a spreadsheet program.
   2. Normalize the wells to the average growth from the no drug plate in a spreadsheet program.
   3. Using spreadsheet software, calculate the inhibition by subtracting the net growth from 1.
   4. Average the columns in the control plate containing only the input gradient in the spreadsheet software.
   5. Calculate the bliss score for each well by the equation:
      Bliss Score = (*well X* + *input column X*) – *Combined well X*
   6. Look for negative scores in wells below the MIC90 of the input.
      NOTE: Negative scores across multiple concentrations would indicate a synergistic interaction.

4. High-throughput Screen with Synergy Prediction Mutants to Identify Novel Synergistic Pairs

1. **Identify synergy prediction mutants**

NOTE: Step 1.5 identified putative synergy prediction mutants. Here, we determine which of these mutants will function best in a high-throughput screen for synergistic drugs. We performed this assay in a Bioscreen machine, but 96 well plate readers should also be acceptable.

1. Grow each putative synergy prediction mutants and wild-type cells in M9 minimal media.
2. Set up the 100 well honeycomb plate (or 96 well plate) with the appropriate growth medium, growth medium + input drug, growth medium + known synergistic partner of the input drug, and growth medium + known non-synergistic drug. Ensure to include vehicle controls.
   NOTE: We recommend four replicate wells for each strain/drug/concentration.
3. Inoculate 1,000 cells per well, including blank control wells.
4. Grow 48 h at 37 °C, with shaking, and read OD_{600} every 20 min.
5. Determine drug concentration and time point that shows the largest growth difference between wild-type and synergy prediction mutant cells in the presence of the input drug and its known synergistic partners. At the optimal time point and concentration, there should not be much growth difference between wild-type and synergy prediction mutant cells when grown in the presence of drugs known to not act synergistically with the input drug (**Figure 3**).

2. **High-throughput screen for synergistic drugs**
   1. Grow wild-type cells and synergy prediction mutant cells in M9 minimal media.
      NOTE: Use appropriate growth medium for organism of interest.
   2. Add drugs from the library to media so each well which contains 100 µL media with small molecules at set concentration which was determined in the previous step. Include blank wells (without cells) and wells with vehicle controls (e.*g.*, DMSO) to account for any growth inhibition by the drug dilution vehicle.
      1. Prepare at least four vehicle control wells per plate, ideally scattered across the plate to account for well position effects. If assays are variable, then include at least one vehicle control well per row/column and use each row/column control well as the control to calculate Z scores for each row/column instead of for an entire plate (step 4.2.5).
   3. Add 2 µL of culture to each well as before. (One plate for wild type bacteria and one plate for predicted synergy mutant bacteria).
   4. Assess the OD_{600} at 0 and 18 h for *E. coli* or 48 h for *C. neoformans*.
   5. Using spreadsheet software, calculate the net growth by subtracting the OD_{600} of blank wells from the vehicle control wells. Identify wells with a Z-score of -2.5 (Z score = mean - 2.5*standard deviation). These wells contain a potential synergist small molecule.
   6. Validate screen hits using the methods outlined in step 3.

Representative Results

Checkerboard assays are a semi-quantitative method for measuring synergistic interactions. The final score output, FICI, determines if a drug combination is considered synergistic (FICI ≤0.5), non-interacting (0.5 <FICI <4), or antagonistic (FICI ≥4.0). **Figure 1** illustrates how to set up the drug gradients in a checkerboard assay. **Figure 2** illustrates common outcomes. Consider growth (purple wells) which display less than 90% growth inhibition. After measuring the OD_{600} of each well in a plate, we normalize everything to the no drug control well. Anything with a normalized value >0.1 (10% of the OD_{600} of the control well) is scored as "growth". FICI scores can vary depending on which well is picked; we select the well that gives the lowest FICI for each plate. In **Figure 2A**, that would be well F9 (columns are numbered, rows lettered), with an FICI = 0.07. In **Figure 2B**, the FICI is calculated from well C3 (FICI = 1.0). For antagonistic interactions, look for the highest FICI score possible. In **Figure 2C**, the FICI is calculated from well A1, which give an FICI of 8.0.

Optimal screening conditions will prevent a high number of false positives from the screen, which saves time and money. When we optimized the synergy prediction mutants for the fungus *Cryptococcus neoformans*, we tested the input drug (fluconazole), four known non-synergistic drugs (caffeine, climbazole, trimethoprim, and brefeldin A), and five of fluconazole's synergistic partners (rifamycin, myriocin, nigericin, rapamycin, and FK506, all discussed in Chandrasekaran, S. *et al.*[18]). Since we discovered these molecules through O2M analysis (sections 1 and 2), we expected the known synergizers to selectively inhibit the growth of synergy prediction mutant cells but not wild-type cells. To maximize the expected growth difference, we tested a range of concentrations for each small molecule, most of which were sub-inhibitory.

Example results are shown in **Figure 3**. A molecule that does not act synergistically with fluconazole, brefeldin A, inhibited wild-type and synergy prediction mutant growth only slightly, and approximately the same amount. Rifamycin (**Figure 3B**), the growth of the synergy prediction mutant (*cnag_03917*Δ) was inhibited, but wild-type cell growth was not. The greatest growth difference was between 32 and 49 h post-inoculation, so the timepoint for screening was in this range. Growth curves of the other synergistic and non-synergistic molecules resembled those of rifamycin and brefeldin A, respectively.

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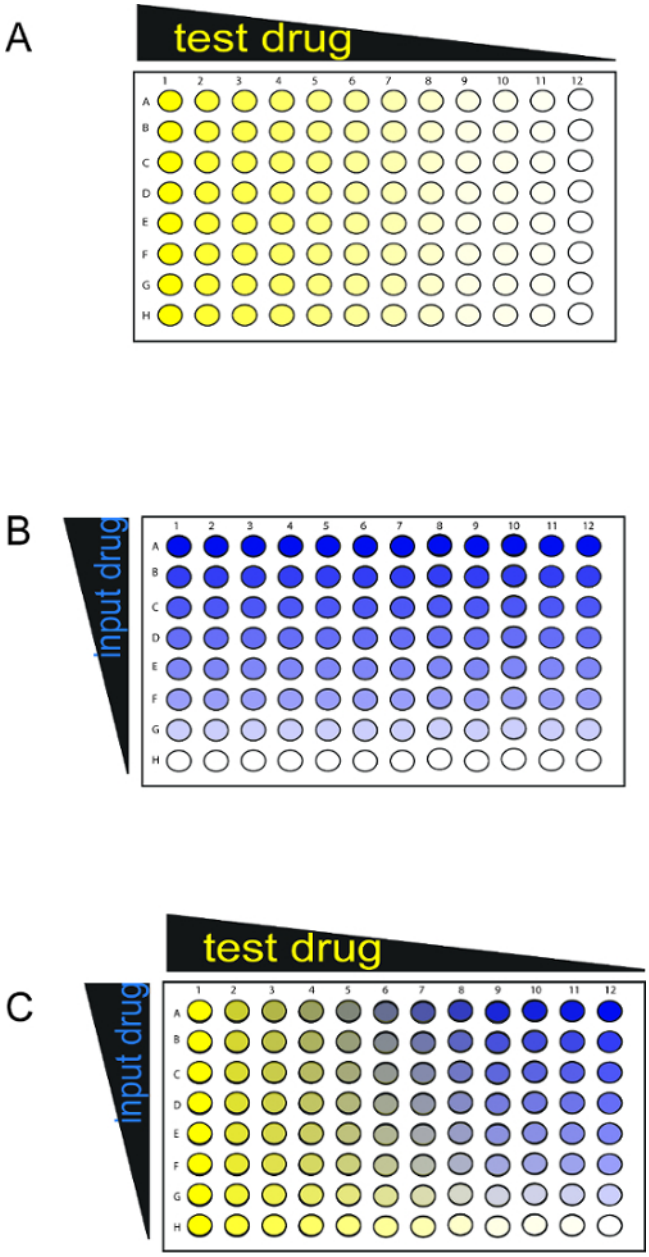

Figure 1: Depiction of Checkerboard Assay from step 3. The test drug and input drug gradients are illustrated in A and B, respectively. The final assay plate should appear as in C. Please click here to view a larger version of this figure.

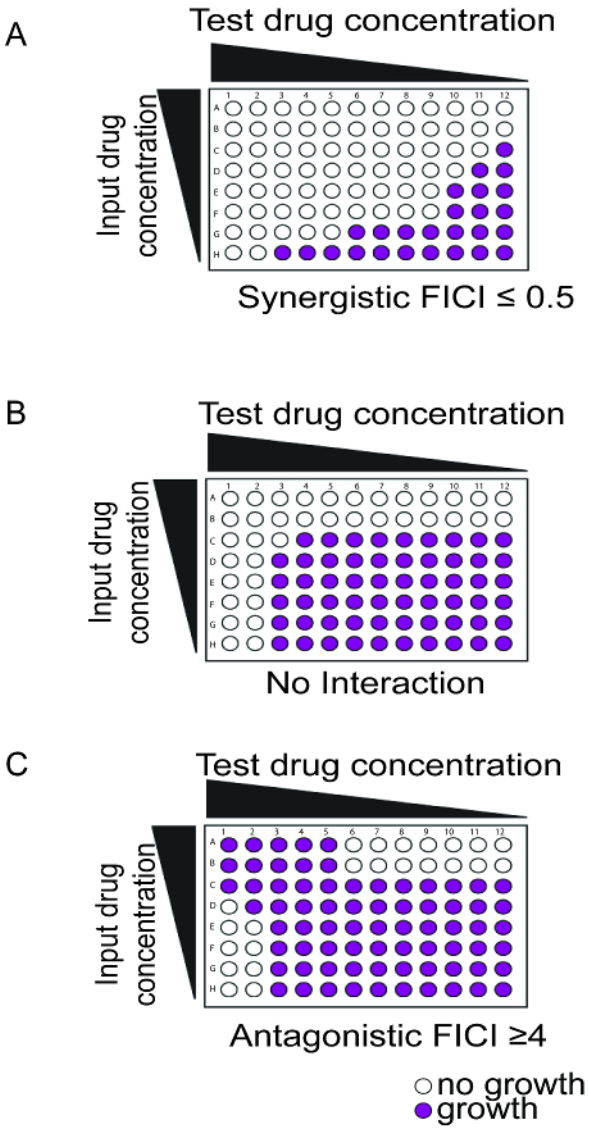

Figure 2: Graphical results of Checkerboard Assay. Illustrations of synergistic, none, and antagonistic interactions are depicted in A, B, and C, respectively. Please click here to view a larger version of this figure.

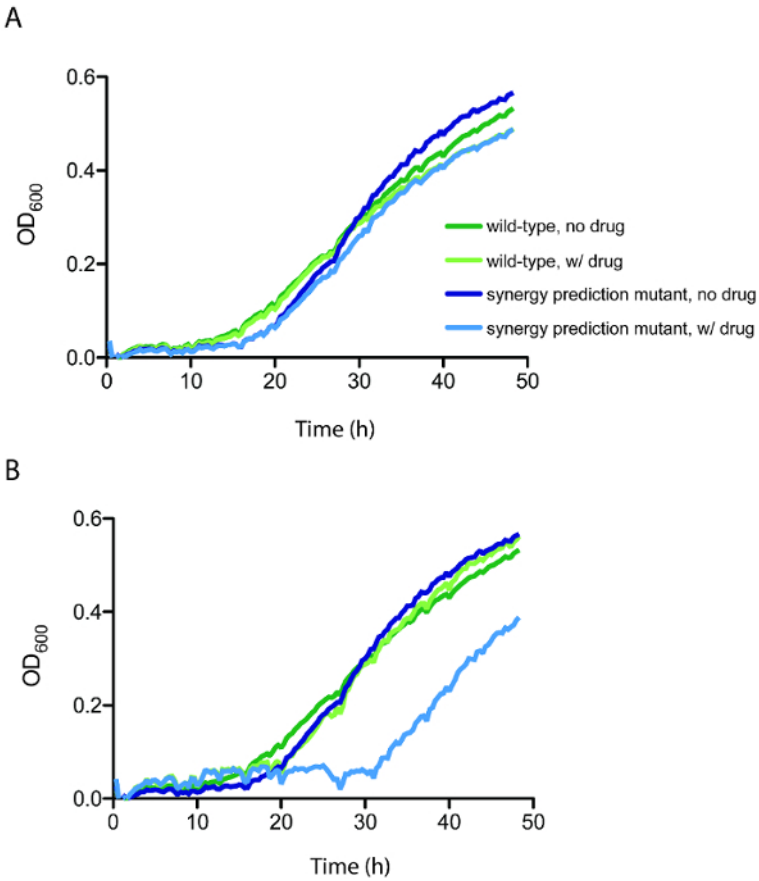

Figure 3: Example data for identifying screening time. (**A**) Wild-type (green) and synergy prediction mutant (blue) of *C. neoformans* grown in the presence of a brefeldin A, a small molecule known to not synergize with fluconazole. Wild-type control (dark green) and drug treated (light green) have a similar difference in growth to the synergy prediction mutant control (dark blue) and drug treated (light blue). (**B**) Wild-type and synergy prediction mutant grown in the presence of rifamycin, a small molecule known to synergize with fluconazole. Wild-type control (dark green) and drug treated (light green) have a smaller growth difference than the synergy prediction mutant control (dark blue) and drug treated (light blue). The greatest difference is observed at 48 h for the known synergistic molecule and the difference is similar at that time point for the known non-synergistic molecule. Please click here to view a larger version of this figure.

Discussion

Synergistic small molecule pairs can be a powerful tool in treating microbial infections, yet they have not reached their full clinical potential because synergistic pairs are challenging to identify. This paper describes a method for identifying synergistic pairs much faster than simple pairwise combinations. By using chemical-genetics datasets, O2M identifies mutants with gene knockouts that can then be used as a readout to screen large libraries of small molecules in order to predict synergistic pairs. The ability to predict small molecules allows for the high scalability of screens, which in turn makes for largescale identification of synergistic partners. After identifying synergistic pairs, one can elucidate the molecular mechanism underlying synergistic interactions, then rationally design additional synergistic pairs[11].

O2M requires a chemical-genetic dataset and a known synergistic pair. Fortunately, chemical-genetics datasets are common for a variety of microbes[19,20,21]. The Brown lab previously demonstrated that O2M can successfully find synergistic pairs in both *C. neoformans* and *E. coli*[11,12]. This demonstrates that the broad impact O2M has as a scalable method that is broadly applicable to a variety of organisms. All steps listed here can be altered for the growth of a different microorganism with relatively similar results, thus making O2M a valuable tool for general identification of synergistic pairs. Several other groups are identifying synergistic combinations from chemical-genetics datasets by different analytical methods, although O2M requires the least programming knowledge of any of these methods. Each method identifies different sets of synergistic antibiotics or antifungals [18,22,23,24], suggesting that a large number of synergistic pairs remain to be discovered. O2M and other synergy prediction methods are also potentially applicable to mammalian systems, including identifying cancer drug combinations.

In sum, this method describes a fast way to screen for synergistic pairs from a chemical-genetics dataset. This method and others help synergistic pairs become a more feasible treatment option in clinics. Additionally, O2M's fast and generalized method proves it a valuable tool when seeking out synergistic small molecule pairs.

Disclosures

The authors have nothing to disclose.

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Acknowledgements

This work was supported by a startup grant from the Department of Pathology, University of Utah to J.C.S.B.

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