## Title
Optimization of drug combinations using Feedback System Control



## Journal
Nature Protocols, 11(2)

## ISSN
1754-2189

## Authors
Nowak-Sliwinska, Patrycja
Weiss, Andrea
Ding, Xianting
et al.

## Publication Date
2016-02-01

## DOI
10.1038/nprot.2016.017

Peer reviewed

# Optimization of drug combinations using Feedback System Control

Patrycja Nowak-Sliwinska[1], Andrea Weiss[1], Xianting Ding[2], Paul J Dyson[3], Hubert van den Bergh[3], Arjan W Griffioen[1] & Chih-Ming Ho[4]

[1]Department of Medical Oncology, Angiogenesis Laboratory, Vrije Universiteit (VU) University Medical Center, Amsterdam, the Netherlands. [2]Med-X Research Institute, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, China. [3]Institute of Chemical Sciences and Engineering, Swiss Federal Institute of Technology Lausanne (EPFL), Lausanne, Switzerland. [4]Department of Mechanical and Aerospace Engineering, University of California, Los Angeles, California, USA. Correspondence should be addressed to P.N.-S. (p.nowak-sliwinska@vumc.nl) or C.-M.H. (chihming@g.ucla.edu).

Published online 14 January 2016; doi:10.1038/nprot.2016.017

**We describe a protocol for the discovery of synergistic drug combinations for the treatment of disease. Synergistic drug combinations lead to the use of drugs at lower doses, which reduces side effects and can potentially lead to reduced drug resistance, while being clinically more effective than the individual drugs. To cope with the extremely large search space for these combinations, we developed an efficient combinatorial drug screening method called the Feedback System Control (FSC) technique. Starting with a broad selection of drugs, the method follows an iterative approach of experimental testing in a relevant bioassay and analysis of the results by FSC. First, the protocol uses a cell viability assay to generate broad dose-response curves to assess the efficacy of individual compounds. These curves are then used to guide the dosage input of each drug to be tested in combination. Data from applied drug combinations are input into the differential evolution (DE) algorithm, which predicts new combinations to be tested *in vitro*. This process identifies optimal drug-dose combinations, while saving orders of magnitude in experimental effort. The complete optimization process is estimated to take ~4 weeks. FSC does not require insight into the disease mechanism, and it has therefore been applied to find combination therapies for many different pathologies, including cancer and infectious diseases, and it has also been used in organ transplantation.**



## INTRODUCTION

Drug combinations are often used to achieve enhanced therapeutic efficacy in the treatment of diseases such as cancer[1], viral infections[2], diabetes, asthma and inflammatory disorders[3,4]. However, it is a challenge to identify optimal drug combinations because of the large number of possibilities; important factors in determining combination efficacy include not only drug selection, but also dosing, administration sequence and timing. An additional problem with combining drugs is toxicity, which is difficult to predict because of the inherent complexity of the underlying biological networks. A challenge in identifying optimal drug combinations is that even a few drugs at several dose levels will end up in a large testing space. For instance, testing ten drugs for cytotoxicity in cancer cells at four different concentrations would lead to the screening of $4^{10}$ (>1 million) possible drug combinations, without taking sequencing or timing into consideration. This would make examining every possibility a laborious, lengthy and costly effort.

Current clinical practice in finding drug combinations is mainly through trial and error. As it is widely accepted that the improvement of disease treatment can be achieved by the combination of drugs, we have developed a method called the Feedback System Control (FSC) technique to systematically search for optimal drug combinations. This drug screening method is based on the integration of experimental results and a search guided by the DE algorithm[5,6]. FSC can save several orders of magnitude in terms of time, cost, effort and numbers of test subjects, as compared with conventional drug-screening methods. The FSC technique[7–17] does not focus on detailed pathway interactions, but it directly focuses on integrative system responses; i.e., the differences between desired and real system responses are used as optimization criteria by a search algorithm, which iteratively drives the system toward a desired phenotypic output.

FSC has been shown to be suitable for use in an array of different applications, such as (i) the inhibition of infectious diseases[18], (ii) the maintenance[12] and differentiation[10] of stem cells, (iii) the control[7] of cancer growth and tumor angiogenesis[14] and (iv) the optimization of the multiple compounds present in herbal medicine[19]. These results have provoked great interest from clinicians, as well as from industry, in applying this approach to the design of new and efficient therapies in a wide spectrum of diseases.

### The FSC platform

The FSC method is based on the closed-loop feedback control process outlined in **Figure 1**. The first step is the definition of an initial set of compounds to be tested. In the next step, broad dose-response curves are generated for each compound in the selected cellular bioassay. The bioassay is selected to provide a phenotypic output response that is used to assess the efficacy of the drugs and drug combinations on overall cell activity. The FSC technique comprises five main components: (i) the system to perform the optimization for, (ii) the inputs for modifying the system's behavior, (iii) the phenotypic outputs of the system, (iv) the search algorithm and (v) a regression method for input-output analysis.

Dose-response curves are used to select the drug-dose input for each compound to be used in combination (**Fig. 1a**). Next, drug combinations are tested on the basis of a cellular bioassay in the selected cell type, which is referred to as the system (**Fig. 1b**). The output response is used as an indicator of the drug combination efficacy *in vitro* (**Fig. 1c**). The FSC optimization is thus based on integrative system responses, wherein the difference between the desired and real system response is used as optimization criterion to be fed into a search algorithm. The search algorithm therefore uses the difference between the results of the tested drug

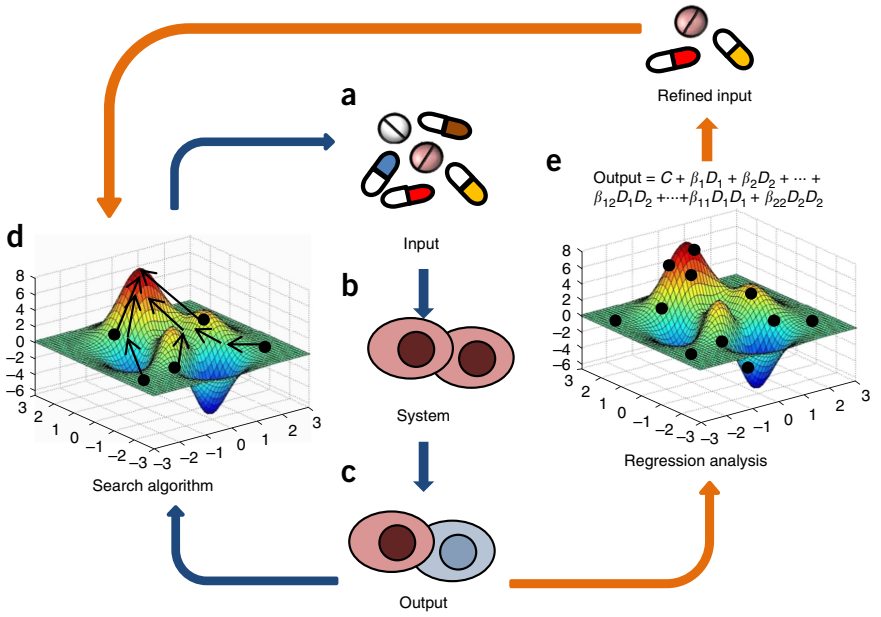


**Figure 1** | A schematic representation of the FSC technique, showing the five main components of the optimization process. (**a**) The input—i.e., drug combinations with defined drug doses. (**b**) The system—i.e., the selected cell type representation of the disease to be studied (**c**). The system output—i.e., the cellular response to the defined drug combination input in the selected cell bioassay. (**d**) The search algorithm that iteratively drives the system output toward the desired response. (**e**) The statistical analysis used for input-output analysis to guide drug elimination.

combinations and the desired optimization goals to predict new combinations to be tested *in vitro*, and to iteratively drive the system to a desired systemic fate (**Fig. 1d**). The DE algorithm was chosen for our protocol. A regression method for input-output analyses can be used to aid in the elimination of antagonistic drugs from the mixture, if the desired system output is not reached or too many drugs are present in the optimized combination (**Fig. 1e**). Each of these components must be defined before starting the optimization; we describe these components in the subsequent sections.

### Experimental design

**Input definition.** A broad selection of drugs that target varying nonoverlapping pathways should be considered. In the example presented in this protocol, nine drugs that target a broad spectrum of endothelial cell signaling pathways were selected for FSC-based screening. This selection covered tyrosine kinase inhibitors, specific anti-angiogenic antibodies, an anti-angiogenic-specific designer peptide, a histone inactivator and a mechanistic target of rapamycin (mTOR) inhibitor (see **Supplementary Note**).

In an optimization targeting a specific cancer cell line, for example, one should consider, but not be limited to, the drugs that target known genetic aberrations or driving mutations in the cell line. This should preferentially include current clinical drugs or drugs in an advanced stage of clinical development. The inclusion of a broader spectrum of drugs and a wider range of drug doses will increase the chances of identifying optimal synergistic drug combinations. An important attribute of FSC is that it can rapidly select a subgroup of efficacious drugs from a large pool of drug candidates. Hence, the initial search should cover a broad spectrum of drug candidates that may well include drugs that were originally not used in treating the target disease. These candidates can serve the goal of possible repurposing of drugs for different applications.

The input drug-dose concentrations to be used in the optimization procedure are defined by first producing a single drug dose-response curve for each of the selected compounds in the selected bioassay. The number of drug doses selected per compound should be decided based on the optimization goal, and it might be limited by the number of compounds being considered in the optimizations. For an initial screen of 5–10 drugs, unless a particular drug shows a steep dose-effect curve, it is appropriate to use three dose levels for each compound, as this is adequate to cover both the linear contributions (owing to single-drug dose change) and the synergistic contributions (owing to drug-drug interactions) of the drugs in the study.

**System definition.** The next step in the optimization is to define the system in which the optimization of a desired behavior will be performed. In the protocol described here, we will focus on an *in vitro* scenario in which the system of interest is an immortalized human endothelial cell line (ECRF24) and the desired output is inhibition of cell viability. Of course, the process is valid for other complex systems both *in vitro* and *in vivo*.

Efficient propagation and maintenance standards for the selected cell type should be implemented. This includes culturing in an optimal medium and proper subculturing (PROCEDURE Steps 1–11). Constant cell maintenance and preparation methods (i.e., splitting ratio and timing, confluence and cell density at the start of the experiment) will help to ensure maximal reproducibility of the bioassay. Depending on the system, a relevant control cell line may be included in the optimization. This allows the optimization to be based on the therapeutic index (i.e., the ratio of the effect on the cancer cell versus that of the healthy matched control cell) of the treatment administered. This facilitates the identification of drug combinations and minimizes the chances of inducing side effects or toxicities.

**Output selection.** An output response must be selected to quantify the response of cells to each drug or drug combination. The output must be able to accurately reflect the phenotypic cell behavior being optimized, and it is therefore important to the overall success of the optimization (e.g., when optimizing anticancer drug combinations, cancer cell viability will be a preferred output over cell migration). To facilitate the FSC screen, it is also necessary that the assay generating the output is robust and that it can be performed reliably and with little variation.

An optimization based on more than one output response can also be performed—i.e., on the basis of both cell viability and cell migration. Alternatively, the effect on multiple cell types may be taken into consideration—e.g., a cancerous and a healthy tissue cell type—to define the therapeutic index, or one can consider

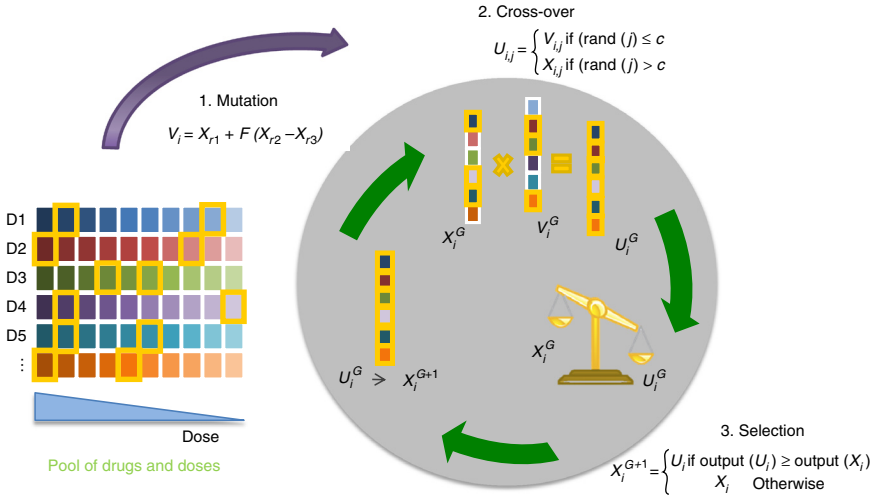


**Figure 2** | Illustration of the differential evolution algorithm. The overall aim is to find the drug combination that yields the best therapeutic effect from an initial pool of drugs tested at certain doses. The first step involves randomly selecting three drug combinations (referred to as $X_{r1,\ r2 \text{ and } r3}$) from the initial pool of drugs and doses, and using them to generate the mutation vectors (termed $V$) based on the equation presented (1). The parameter $F$ represents how strongly the difference of the two randomly selected vectors will be weighed when added to the first randomly selected vectors during the mutation step. $F$ will have an effect on how quickly the algorithm converges, as it is one of the key factors affecting how much trial combinations will vary from the target combinations. A good starting value of 0.5 for $F$ has been suggested[46]. In the cross-over step (2), a new set of combinations ($U^G$) is designed by including randomly chosen elements from the original vectors $X^G$ and the mutation vectors $V^G$. The random selection is performed based on comparing the value of a randomly generated number to the cross-over constant ($C$; equation in step 2). The cross-over constant essentially determines how many of the components from the mutated combination will be included in the new trial combination ($U^G$). The cross-over constant will also have an effect on how much trial vectors vary from target vectors and therefore have an effect on the rate of convergence of the algorithm. The value of $C$ can range from 0 to 1. A value of 0.5 for $C$ is implemented[47]. Hence, $U^G$ will generally consist of 50% drug-dose combinations from each $X^G$ and $V^G$. In the last step, which is the selection step, the new combinations ($U^G$) are experimentally tested and their performance is compared with the original vectors ($X^G$). The vector that performs better becomes the next original vector ($X^{G+1}$, equation in step 3). The entire process is then repeated (i.e., performed iteratively).

multiple cells with different genetic characteristics. In such an optimization, all outputs must be incorporated into a single overall output response, which is provided by an equation integrating all the outputs to be optimized. The overall output must be able to describe the efficacy of each drug combination. The most common way to integrate all the outputs into one formula is to assign a weighing factor (indicating the relative importance of the output) for each output and to summarize the weighted output together. The decision on the weight factor for each output response needs to reflect the relative significance of these output responses. This requires prior mechanistic insight into the process being optimized, and it should therefore be determined on a case-by-case basis.

**Stochastic search algorithm.** Although many stochastic algorithms could be implemented in the FSC technique, we describe the use of the DE algorithm[20,21]. The DE algorithm is a population-based stochastic search algorithm. DE has been previously proven to adequately and efficiently search for the effective drug combinations after only 10–20 iterations of experimental efforts by testing 15–20 different drug combinations per iteration[12]. DE is a genetic algorithm, which was originally developed by Storn in 1995 (ref. 20). Similarly to other evolutionary algorithms, DE is a stochastic simulation of biological evolution through iterative updates that increase individual adaptation to the surviving conditions requested by the environment (**Fig. 2**). The DE-guided search strategy drives the exploration of the entire search space, while avoiding becoming trapped in a local optimum.

In each iteration of the DE algorithm, a new set of trial drug combinations ($U^G$), which are generated by the steps of mutation and cross-over, are tested and compared with the original set of drug combinations ($X^G$; **Fig. 2**). Drug combinations with better overall efficacy are selected to form the next iteration of target drug combinations ($X^{G+1}$), whereas the ones with worse overall efficacy are abandoned. This iterative process is repeated until the optimal drug combination is identified or no further significant improvements in cell viability inhibition can be found. The latter is called an improvement-based stopping criterion[22] (i.e., the improvement in the output value of the best-performing drug combination, the average improvement in all combinations or the number of trial combinations (referred to as '$U$' in **Fig. 2**) is accepted over the target combinations (referred to as '$X$' in **Fig. 2**) in each iteration). If the optimal drug combination identified by the DE algorithm is not desirable (i.e., it contains too many compounds or does not have a high enough efficacy), one should proceed to data modeling and analysis.

**Data modeling.** The data from all previous FSC-based studies have demonstrated that the drug-dose combination versus drug efficacy landscape surface is smooth, which permits the DE algorithm to rapidly identify the global optimum.

When starting with a sizable number of test compounds, regression analysis is used to select the most potent drug contributions, as well as to indicate synergistic, additive or antagonistic drug interactions. Regression analysis can be performed in MATLAB[23] (**Box 1**; **Supplementary Software** using the file 'RegressionAnalysis.m'), R (**Supplementary Software** using the file 'RegressionDemo.R') or other appropriate programs. By using all data points obtained during the preceding optimization, a stepwise regression model[24] can be generated, which mathematically describes the cellular activity (in terms of the selected bioassay output) in response to the drug combinations administered to the cells (i.e., the system input). The second-order regression model includes terms that describe the contributions of each single drug (first and second order, $\beta_i$ and $\beta_{ii}$, respectively), as well as those of the two-drug interactions ($\beta_{ij}$)[7,8,14,25] to the overall cell output response.

## Box 1 | Data modeling and analysis ● TIMING 2 h

▲ CRITICAL STEP The data modeling and regression analysis step is only needed if the primary optimization does not identify a desired optimal drug combination (i.e., there may be strong antagonism between certain agents) or if drug elimination is desired.

1. Open the code in order to perform linear regression analysis in either MATLAB or R programs (**Supplementary Software**). Steps 2–4 below describe how to implement the MATLAB code, whereas the implementation of the R code is described in the **Supplementary Tutorial**.
2. Download the MATLAB file called 'RegressionAnalysis' from **Supplementary Software** and open it in MATLAB.
3. Run MATLAB code (press F5 on the keyboard or select Debug and select 'Run RegressionAnalysis.m').
4. Insert the data for the regression analysis in the command window. The drug combinations should be entered as a matrix of dimension [$D \times N$] surrounded by brackets (with $D$ representing the number of drugs and $N$ representing the number of drug combinations), where each column represents a drug combination. The efficacy of each drug combination should be entered as an [$N \times 1$] vector.

**? TROUBLESHOOTING**

5. Analyze the results of modeling in the command window in the form of the regression coefficients. Note that two models are generated: the first model containing all data points and the second model with outliers removed based on the criteria of Cook's distance. Two figures are automatically generated for each regression model: the real versus predicted observations, and the residual plots (**Fig. 3**).

▲ CRITICAL STEP For the removal of outliers, the code provides three options. MATLAB code can be manipulated in the '.m' file provided in the **Supplementary Software**. The file can be opened in the file editor by double-clicking on the '.m' file from the 'Current Folder' (generally located above the command window). Lines of code can be deactivated by inserting a '%' at the beginning of each line (nonactive lines of code will appear in green text). To remove only the largest outlier, activate line 71 of the code and deactivate line 66; to remove all outliers with a cook's distance greater than three times the average Cook's distance value, activate line 66 and deactivate line 71; or to remove no outliers from the data set, deactivate both sections of the code (lines 66 and 71).

6. Analyze regression coefficients and concentrations to eliminate the least prominent compounds from the search. If the desired goal is to minimize the system output, negative regression coefficients (first-order and second-order single drug terms, and two-drug interaction terms) represent the more desirable drug activity. If the optimization goal is to maximize the system output, positive regression coefficients represent desirable drug activity.
7. Once the compounds have been eliminated, return to the optimization process (Step 43 to re-define drug doses and Steps 44–58 for DE optimization) using the refined set of drugs.
8. If optimal drug combination is identified by Step 58, stop the optimization; otherwise, repeat the data modeling and analysis steps described in this box.

A complete second-order regression model is represented by equation 1:

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ represent the coefficients of the intercept, linear, quadratic and bilinear (or interaction) terms, respectively, $y$ represents the response variable (the output), $x_i$ and $x_j$ are independent variables (the inputs, which represent the drugs) and $\varepsilon$ is an error term with a mean close to zero[26].

The main assumptions of a linear regression model should be verified—i.e., weak exogeneity, linearity, constant variance, independence of errors and lack of multicolinearity. This will ensure the model's accuracy and the reliability of model-based predictions. Confirmation of model accuracy requires moderate statistical knowledge, and it can be performed on the basis of the assessment of the following analyses (**Fig. 3**): the fitted efficiency (based on $R^2$ value and a plot of the experimental versus the fitted data), residual analysis (residual distribution, normal probability plot and residual histogram) and analysis of outliers (Cook's distance). Finally, the predictive potential of the model is to be verified by testing predicted drug combinations *in vitro*.

Several improvements to the original FSC system have evolved. After exploring the iterative search strategy in FSC, we discovered that the drug combination efficacy is related to the applied drug doses by a quadratic algebraic response surface[7]. **Figure 4** provides a visual representation of the response surfaces between the system output ($z$ axis, labeled output) and the varying dose of only two drugs in the combination ($x$ and $y$ axis, labeled inputs 1 and 2). The smoothness of these surfaces is the fundamental reason for the ability of FSC to identify the most potent drug combination from a large parameter space with only about ten iterations, thus making it an efficient drug combination screening method[18,24]. This is an important observation, as it is expected to markedly simplify the *in vitro* to *in vivo* translation in which differences in pharmacokinetics and dynamics may strongly perturb the optimal combination.

### Applications of FSC

As FSC facilitates the identification of optimal drug combinations without requiring mechanistic insight[8], it is attractive for many applications. It has been used for the identification of optimal drug combinations in various complex biological systems (**Fig. 5**).

FSC has previously been used in drug optimizations for the treatment of infectious disease, including vesicular stomatitis virus (VSV) infection in NIH 3T3 fibroblast cells[15] and Kaposi's sarcoma–associated herpes virus re-activation[17]. HIV, hepatitis C virus and influenza infections have been shown to be effectively treated by combinations of antiviral drugs. For instance, Ding *et al.*[18]

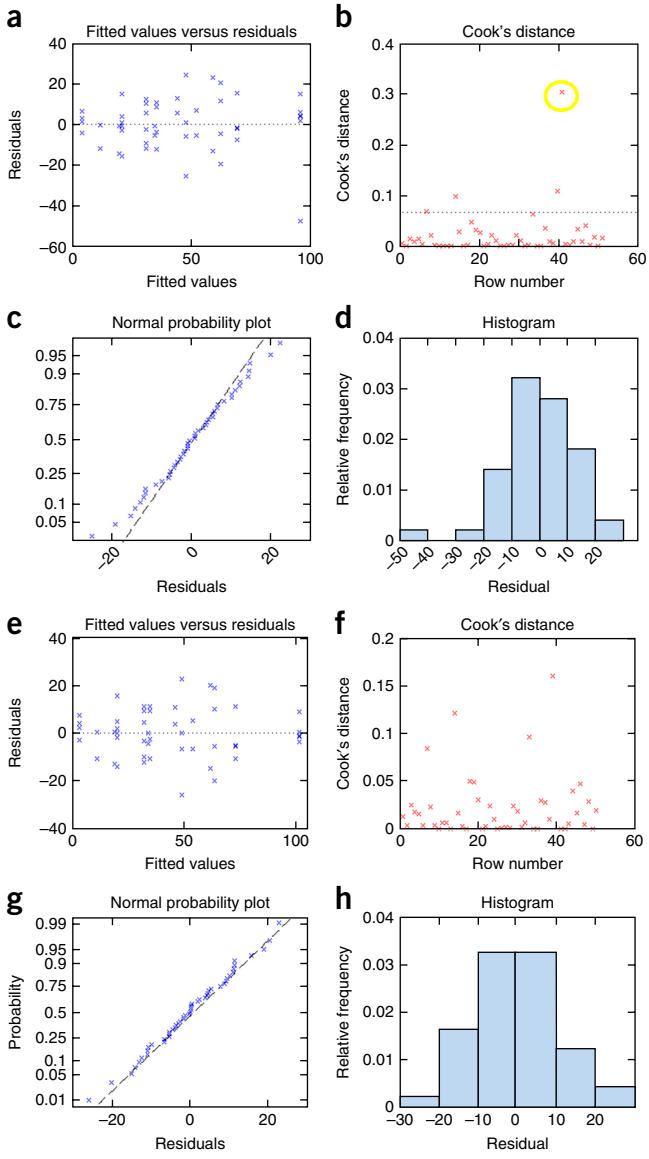


**Figure 3** | Regression analysis of combinatorial data. (**a**–**f**) Plots for residual analysis of a data set are presented before (**a**–**d**) and after (**e**,**f**) the removal of an outlier, including residual plots (**a**,**e**); Cook's distance plots (**b**,**f**; outlier indicated by a yellow circle in **b**); normal Q-Q plots (**c**,**f**); and residual histograms (**d**,**h**). Data outliers can be identified based on the plot of Cook's distance (**b**,**f**). The largest outlier or any data points with a Cook's distance greater than three times the average Cook's distance (represented by the dotted line in **b**) can be eliminated. In this example, removal of the data point with the largest Cook's distance improved the residual plots for the data set.

showed that FSC rapidly identified optimal drug combinations that inhibit herpes simplex virus-1 infection, by testing only several hundred drug combinations.

One of the important problems in stem cell–mediated regenerative medicine is the development of defined culture systems for the maintenance of clinical-grade human embryonic stem cells. The FSC technique has proven to be helpful in the optimization of the conditions for the maintenance[15] or differentiation[10,12] of stem cells. Another successful application of the FSC method was in the optimization of a flavonoid-based herbal drug combination[19]. This strategy allowed an effective drug combination to be identified, which was composed of four synergistic drugs with doses reduced by approximately tenfold as compared with those found in the original herbal extract.

In another example, FSC was used to establish the differential response of cancer (non-small-cell lung cancer) and normal cells to combinations of three chemical agents[7]. The authors found a combination of drugs that led to a significant difference in the responses of different cell types, clearly increasing the differential targeting of cancer and normal cell lines with the best drug combinations.

A similar goal was achieved by Wang *et al.*[8], in which differential response of breast cancer and multiple control cells was optimized. The final drug combination in that case consisted of three nanodiamond-immobilized drugs and one unmodified drug. Again, the therapeutic efficacy and selectivity of the optimized combination was significantly higher for the best mixture as compared with that of the individual drugs by themselves.

Recently, we applied the FSC technology to the identification of an effective low-dose angiostatic drug combination[14]. After a few optimization cycles—i.e., only a few hundred measurements in total—a second-order equation was fitted to the 'landscape' of drug-combination input and drug-viability output data. The coefficients in the 'best-fit' equation permitted the elimination of several drugs. With the remaining most active drugs, and a few more measurement cycles, we then identified the final optimal drug mixture containing only three drugs. These interacted synergistically[27], so that only very low concentrations are needed for effective antiangiogenic activity. Interestingly, the combination of drugs enhanced the specificity for endothelial cells. The *in vivo* validation showed that the optimized drug combination was quite effective in two preclinical models of tumor growth inhibition as compared with that of individual drugs by themselves, which were administered at much higher doses.

In all the applications mentioned above, only testing of a small percentage of total drug combination possibilities was required to accurately establish a predictive model that was capable of

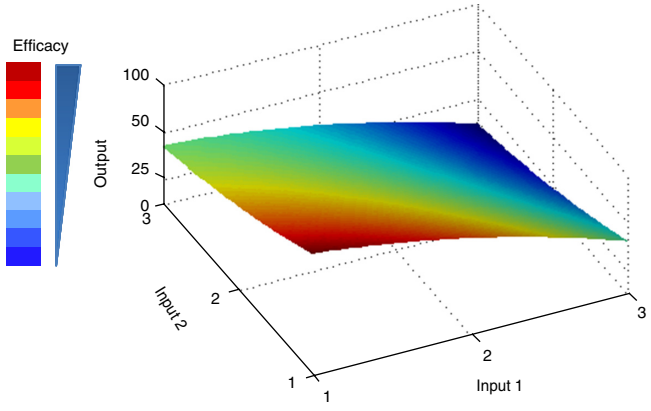


**Figure 4** | Schematic drawing of a smooth response surface. We examined drug-dose-response surfaces using the data acquired from the FSC optimization process in various biological systems (e.g., cancerous cells). In all of the systems that we have previously studied, smooth response surfaces were observed.

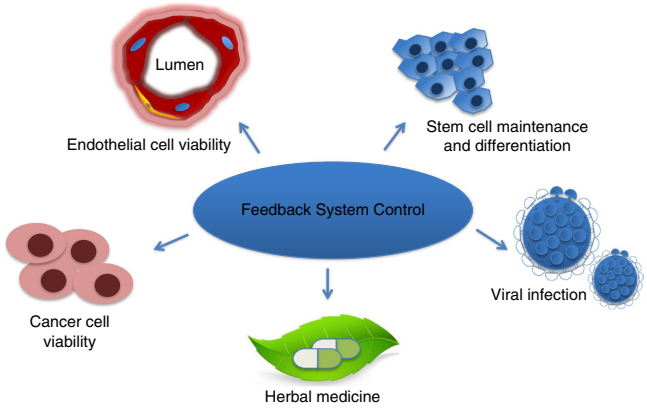


**Figure 5** | Schematic drawing of reported FSC applications. FSC technology has been instrumental in endothelial cell viability inhibition, stem cell maintenance and differentiation, viral infections, herbal medicine or cancer cell viability inhibition.

simulating the effect of all possible combinations in the domain of the drug concentrations investigated.

### Comparison with other methods

Over recent years, there has been a growing interest in the development of methods for identifying optimal drug combinations[28–30]. In a clinical setting, drug combinations are still mainly selected on the basis of empirical approaches, which are largely guided by clinical experience with individually administered drugs[31,32]. However, many optimization methods have been and are being evaluated preclinically[29,33,34]. They can be classified as follows:

(i) Exhaustive searches—testing all possible drug combinations with a high-throughput screening technique[35]. This approach is limited, as it requires extensive labor, time and cost, which can be prohibitive when the number of drugs and the dose levels increase. As a result, most studies are limited to only testing pairwise combinations of drugs[36].

(ii) Statistical approaches—linear combinations of known input-output relations and desired phenotype are developed, neglecting the nonlinearity in biological networks. In these models, the system is treated as a black box, and it does not require a complete characterization of the biological networks[37,38].

(iii) Model-based combinations in which biological measurements are used to build explicit models of a target network using simulations[39,40]. This approach seems to be one of the most successful in multidrug design. It should, however, be emphasized that we do not fully understand most drugs and their pathways and targets, thus limiting this approach.

(iv) A biological search algorithm. In this case, model-based predictions of effective perturbations are combined with a closed-loop iterative experimental search[34].

It is important to note that modeling cell behavior is limited by the complexity of the cellular signaling pathways, and in cancer, by inter-patient and intratumoral heterogeneity[41]. Although it is possible to make generalized conclusions about cell mechanisms and their interactions on the basis of mathematical models, these models are inherently constrained by the information given to the system and the assumptions used in generating the models.

The FSC technique is not an algorithm. The second-order algebraic drug response surface, equation (1), is the mathematical foundation that seems to be verified by the 'good fits' found in all systems in which this has been tested up thus far. It is the reason for the ultra-effective searches for optimal drug combinations with a small number of converging iterations. In each iteration, a small number of drug combinations are experimentally tested to measure cellular (or organism) phenotype in response to a drug combination. The Gur-Game algorithm or a genetic algorithm generates new candidate combinations to be tested, on the basis of the previous results. The limitation of statistical model-based techniques is that they attempt to approximate the control landscape using training data and then optimize the approximated response, whereas the FSC moves on a simple quadratic algebraic surface and therefore efficiently reaches the optimal value with a small number of iterations. The model-free method is particularly advantageous in dealing with a biological system, which involves a large set (≥10) of drug candidates, in which traditional modeling would require a large amount of data to provide a robust model. Another major advantage of the FSC approach is that it is phenotypically driven, and therefore it does not require mechanistic information about the system, as is required for model-based approaches. Moreover, the response surfaces show a relatively smooth response when doses of the given drugs are varied. In some cases, we have found that a large reduction in the dose of one of the drug in a combination will not proportionally decrease its efficacy. This finding implies that the radius along this drug dose and the efficacy on the smooth surface is fairly large.

### Advantages of FSC

The approach presented here provides a reliable platform for rapid and effective drug combination screening. This can be attributed to several unique features of the approach.

First, the method is phenotypically driven unlike many other approaches, which are genotypically driven. This greatly reduces the amount of background and mechanistic information that needs to be known about the system before the start of the optimization. It also minimizes the chances that the search is led astray because of incorrect or incomplete information. In addition, as it is phenotypically driven, it may increase the likelihood of identifying unexpected drug synergies. This may also lead to drug repurposing, which can allow for new effective therapies while bypassing the time-consuming and costly process of new drug development. Moreover, through the identification of synergistic drug combinations, highly effective combinations using reduced drug doses will hopefully reduce the chances of side effects or toxicity.

Second, this approach combines the use of a search algorithm and regression modeling to aid in the assessment of drug combinations and the elimination of antagonistic mixture components when needed. The use of these two approaches allows for more rapid exploration of the search space (generally an optimum can be identified after exploring $<0.2\%$ of the entire search space). The use of the DE algorithm also aids in the analysis of the nonlinear complex system by searching in a manner that incorporates both a stochastic and an improvement-based guided

search based on the population dynamics. Unlike competing methods, the computational complexity and experimental data required for effective analysis of the search space does not grow exponentially when more drugs or drug doses are included in the search. This allows for the possibility to effectively explore increasingly complex problems.

In all studies conducted so far, we have always been able to identify synergistic drug combinations. Among these were both expected and unexpected synergies. The main challenge is to identify the optimal synergistic combination from an initial selection of drugs. The combined use of the search algorithm and regression analysis drives the search toward the optimal region of the search space corresponding to the optimal activity, which subsequently leads to the elimination of antagonistic drugs. The probability of identifying novel synergies is mainly dependent on the initial experimental design and starting conditions (i.e., drug and dose selection). Data modeling analysis helps to identify antagonistic drug mixture components, and it will therefore aid the user to re-define the experimental design in the case that synergies are not identified. Subsequently, the refined search is designed to identify the synergies, once noise presented by other drugs has been removed from the system.

Altogether, FSC allows rapid converging upon an experimentally verifiable optimal drug combination[15], and it can facilitate the development of new drug combination from *in vitro* to *in vivo* preclinical models to clinical testing.

**Limitations**

- If experimental error or variation in the experimental bioassay is too large, it will prevent the algorithm from being able to identify the optimal combination.
- Visualization of drug interactions by response surfaces is limited to 3D representation of a multidimensional surface.
- The discretization of drug doses (i.e., the selection of distinct drug doses) can result in limitations. This may be the case in high-dimension optimizations, in which many drugs are considered, thus limiting the number of doses that can reasonably be included for each drug. Alternatively, if the sensitivity of the system is unknown and too few drug doses are used, information about drug interactions may be lost[30]. We address this limitation by producing dose-response curves for each compound.
- For initial drug selection, the possibility exists that synergistic interactions within the selected compounds do not exist. In this case, the user should consider performing a new optimization with a wider initial selection of compounds or other drug concentrations.

**Level of expertise needed to implement the protocol**

The FSC protocol requires intermediate cell culture knowledge and skills, moderate understanding of statistical analysis and regression modeling, as well as basic understanding of MATLAB and the ability to implement the MATLAB or R codes provided.

## MATERIALS

**REAGENTS**

- DMEM (1×) + GlutaMAX-I (Gibco, cat. no. 31966-021)
- RPMI 1640 medium (1×) + GlutaMAX-I (Gibco, cat. no. 61870-010)
- DMSO (99.9%; Sigma-Aldrich, cat. no. D8481)
- FBS (Sigma-Aldrich, cat. no. F2442)
- Gelatin solution type B, 2% (wt/vol) in $H_2O$ (Sigma-Aldrich, cat. no. G1393)
- PBS, pH 7.4 (1×) without $Ca^{2+}$ and $Mg^{2+}$ (Gibco, cat. no. 10010-015)
- Trypsin-EDTA, 0.05% (wt/vol) (1×; Gibco, cat. no. 25300-054)
- Penicillin-streptomycin (penicillin 10,000 IU/ml; streptomycin 10,000 µg/ml; Amimed, cat. no. 4-01F00-H)
- CellTiter-Glo luminescent cell viability reagents (Promega, cat. no. G7571)
  **! CAUTION** The reagent is hazardous; avoid contact with body parts.
  **▲ CRITICAL** Avoid light exposure.
- Appropriate cell line. This procedure is optimized for use with ECRF24 (human immortalized umbilical vein endothelial cell line; VU Medical Center, available on request). It can be adapted for use with other cell lines
  **▲ CRITICAL** For these experiments, non-immortalized endothelial cells can be used as well **! CAUTION** If you are using cell lines in your research, they should be regularly checked to ensure that they are authentic and not infected with *Mycoplasma*.

**EQUIPMENT**

- $CO_2$ cell culture incubator (Sanyo, model MCO-18AC)
- Inverted microscope (Leica, cat. no. DMI3000)
- Sterile biosafety cabinet (Skan AG, model VSA-180)
- Cell culture flask, T75 (TPP, cat. no. 90075)
- Cell culture plates, 96 wells, sterile, flat-bottom (Corning, Costar, cat. no. 3596)
- Opaque-walled, flat-bottom 96-well cell culture plates, sterile (Corning, Costar, cat. no. 3603)
- Sealing film, 10 cm × 38 m (Parafilm M, cat. no. 52858-000)
- Sterile serological pipettes, 2 ml (VWR International, cat. no. 612-1243), 5 ml (cat. no. 612-1248) and 10 ml (cat. no. 612-1245)
- Eppendorf Safe-Lock tubes, 1.5 ml, colorless (Eppendorf International, cat. no. 0030 120.086)
- Eppendorf Easypet 3 (Eppendorf AG, cat. no. 4430000.018)
- Multichannel Pipetman Neo (Gilson, cat. no. P200)
- Sterile filter tips, 200 µl (StarLab, cat. no. S1111-0706)
- Luminometer (Tecan Infinite F500)
- Orbital plate shaker (Thermo Scientific, cat. no. 88880023)
- Premium aluminum foil (VWR International, cat. no. 89107-732)
- Sterile filters (Cell Tricks, cat. no. 04-004-2328)
- MATLAB version R2012a or higher (can be purchased from MathWorks)
- R version 3.2.1 (×64) (can be downloaded from http://www.r-project.org)

**REAGENT SETUP**

**Cell medium DMEM/RPMI (1:1), supplemented with 10% (vol/vol) FBS and 1% (vol/vol) antibiotics** Under laminar flow, combine 450 ml of sterile DMEM (1×) + GlutaMAX-I and 450 ml of sterile RPMI 1640 medium (1×) + GlutaMAX-I, and then add 90 ml of sterile FBS and 10 ml of sterile penicillin-streptomycin (penicillin 10,000 IU/ml; streptomycin 10,000 µg/ml). Store the medium at 4 °C for up to 3 weeks. **▲ CRITICAL** The ECRF24 cell line grows substantially better in cell medium DMEM/RPMI mixture (1:1) than in DMEM or RPMI alone.

**Gelatin solution, 0.2% (wt/vol)** Dilute 2% gelatin solution in sterile $H_2O$ to make a 0.2% (wt/vol)) solution. The stock solution is sterilized by filtration by using a 0.22-µm membrane filter. Store the solution at 4 °C for up to 2 months.

**CellTiter-Glo (CTG) solution** Thaw the CTG buffer solution at room temperature (~21 °C) in the dark, and mix it gently until it is dissolved with the CTG substrate to prepare CTG solution. Store the solution in the dark at −20 °C until use. This reagent can be frozen and thawed up to ten times, losing < 10% of its activity.

## PROCEDURE

### Cell culture and passaging ● TIMING 20–30 min

**1|** Precoat the cell culture flask with 0.2% (wt/vol) gelatin solution. To do this, add 2 ml of gelatin solution to a T75 cell culture flask, and tilt the flask from side to side until the entire bottom of the flask is coated. Incubate the plastic flask with gelatin solution for a minimum of 20 min in the incubator (37 °C).

**2|** In the meantime, prewarm trypsin solution, cell culture medium (supplemented, refer to Reagent Setup) and PBS in a water bath at 37 °C for 10 min.

**3|** Aspirate and discard the culture medium from the ECRF24 cells in the tissue culture flask in a sterile laminar flow cabinet.

**4|** Wash the cells twice with 5 ml of prewarmed PBS.

**5|** Add 2 ml of trypsin and incubate for ~2–5 min at 37 °C.

**6|** Remove the remaining 0.2% (wt/vol) gelatin solution from the precoated flask prepared in Step 1 by standing the flask upright and pipetting away the gelatin solution that falls to the bottom of the flask (this gelatin solution can be reused). Place the flask to one side.
**? TROUBLESHOOTING**

**7|** Verify cell detachment with an inverted microscope.
▲ **CRITICAL STEP** The time for enzymatic detachment of cells can vary between cell lines, and it should be monitored carefully. Leaving cells in the presence of trypsin for too long may affect cell viability.

**8|** Stop the reaction by adding 2 ml of prewarmed, supplemented cell culture medium while working in the laminar flow cabinet.

**9|** Mix the cells gently and transfer a selected fraction of cells to the prepared gelatin-coated T75 cell culture flask from Step 6. A cell passage ratio between 1:3 and 1:4 is recommended twice per week.
▲ **CRITICAL STEP** Mixing of cells should be thorough and gentle to ensure disaggregation of cell clumps.
**? TROUBLESHOOTING**

**10|** Fill the flask with additional prewarmed, supplemented cell culture medium to reach a final volume of 15 ml.

**11|** Mark the cell culture flask appropriately with cell type, passage ratio, date and experimenter's initials; incubate the cells at 37 °C until use.
▲ **CRITICAL STEP** It is better to avoid allowing the cells to reach 100% confluency, as this may reduce viability or cause the cells to leave the exponential growth phase.
**? TROUBLESHOOTING**

**Cell seeding in a 96-well plate ● TIMING 40 min**
**12|** Prewarm trypsin, supplemented cell culture medium and PBS in a water bath at 37 °C for 10 min.

**13|** Prepare a 96-well plate by precoating each well with 30 µl of 0.2% (wt/vol) gelatin solution. Gently tap the edges of the plate to ensure that the wells are completely covered. Incubate the plate for 20 min at 37 °C.

**14|** Fill the outer wells (wells A1–A12; B1; B12; C1; C12; D1; D12; E1; E12; F1; F12; G1; G12 and H1–H12) of the flat-bottomed, 96-well, cell culture plate with 100 µl of PBS in the laminar flow cabinet. This procedure will prevent excessive evaporation in the plate and edge effects.

**15|** Take a flask of cells at ~80% confluency (from Step 11) and dissociate the cells (from Steps 2–5, 7 and 8) to transfer them to a sterile 15-ml tube.

**16|** Determine the cell concentration (cells per ml) by using a cell counting chamber or Coulter counter according to the manufacturer's guidelines.
▲ **CRITICAL STEP** Minimize the time that the cells are in suspension.

**17|** A cell seeding density of 10,000 cells per well (or 100 µl per well at a density of 0.1 million cells per ml) is recommended. Dilute the cell suspension prepared in Step 15 to the desired concentration with prewarmed, supplemented cell culture medium.

**18|** Remove additional gelatin solution from the 96-well plate prepared in Step 13 by tilting plate and pipetting out additional liquid using a multichannel pipette.

**19|** Seed the cells with a multichannel pipette into the interior wells of the 96-well cell culture plate (rows B–G, columns 2–11) while continuously mixing the cell suspension to ensure that the desired number of cells is suspended in a volume of 100 µl of cell culture medium.

**20|** Label the 96-well cell culture plates by noting the cell line, plate number, cell density, experimenter's name and the date of preparation.

**21|** Incubate the seeded 96-well cell culture plates at 37 °C for 24 h.

**Drug stock and aliquot preparation ● TIMING 10 min per drug**

**22|** Weigh out the desired weight of the given compound using an analytical balance.

**23|** Add the required amount of drug solvent to the weighed out drug volume taken from the previous step, while working in the sterile laminar flow cabinet. Dissolve the drug in the solvent.
▲ **CRITICAL STEP** Ensure that the solvent concentration in the final working solutions does not influence the readout (i.e., cell viability) or include appropriate controls.
**? TROUBLESHOOTING**

**24|** Divide the stock solution into aliquots with a minimum liquid volume of 20 µl (to minimize the effects of solvent evaporation) in sterile Eppendorf Safe-Lock tubes.
▲ **CRITICAL STEP** Ensure that the volume of stock solution is sufficient for each experiment.

**25|** Carefully mark all aliquots with the drug name, stock concentration and preparation date, and then store them at the appropriate temperature until use. For drugs that have a short-lived stability in solution, prepare stock solutions directly before use (according to Steps 22 and 23).
▲ **CRITICAL STEP** Carefully monitor the date of drug stock preparation and storage temperature.
■ **PAUSE POINT** Stock solutions can be stored according to the manufacturer's recommendations.

**Cell viability assay ● TIMING 2 + 72 h for incubation time per iteration**

**26|** Prewarm supplemented cell culture medium and PBS in a water bath at 37 °C for 10 min.

**27|** Prepare the test solutions by adding the desired drug at the appropriate concentration to the prewarmed supplemented cell culture medium.

**28|** Place the seeded 96-well cell culture plate from Step 21 in the laminar flow cabinet.

**29|** Aspirate and discard the cell culture medium and wash the cells once with prewarmed PBS.

**30|** Administer 50 µl of prepared test solution per well.
▲ **CRITICAL STEP** Each test plate should contain control wells, and each condition should be performed at least in triplicate.

**31|** Incubate 96-well cell culture plates at 37 °C for 72 h.

**32|** After 72 h, remove the plates from the incubator and allow the contents to equilibrate to room temperature for 30 min.
▲ **CRITICAL STEP** Before starting the readout, visualize the cells under an inverted microscope. Check for drug precipitates and monitor the morphology, as well as the confluency, of the cells in the control wells.

**33|** In the meantime, prepare (refer to Reagent Setup) and add 50 µl of CTG solution to each well using a multichannel pipette in order to lyse the cells. Cover the plate with aluminum foil to protect the well contents from light.

**34|** Mix the plate contents for 2 min on an orbital shaker to allow completion of cell lysis.

**35|** Store the plate at room temperature for 10 min to allow the luminescent signal to stabilize.

**36|** Transfer the liquid to the opaque-walled 96-well cell culture plates, while preventing the introduction of bubbles to the solution by reverse pipetting.
▲ **CRITICAL STEP** If the cells are already cultured in opaque-walled 96-well cell culture plates, this step is not necessary.

**37|** Record the luminescence signal using a luminometer. The manufacturer provides the instrument settings. It is recommended to use an exposure time of 0.25–1 s per well.
▲ **CRITICAL STEP** Limit light exposure, as the CTG reagents are light-sensitive.
▲ **CRITICAL STEP** The plate should be compatible with the luminometer used.
■ **PAUSE POINT** Data can be stored and analyzed at a later time point.

**Dose-escalation study of individual compounds ● TIMING 72 h per experiment**
**38|** Prewarm the supplemented cell culture medium at 37 °C for 10 min.

**39|** Prepare and label sterile Eppendorf Safe-Lock tubes.

**40|** Prepare serial drug dilutions from the stock, as prepared in Steps 22–25.
▲ **CRITICAL STEP** The range of dilutions tested should cover the maximal possible dose range of the cell response. Note that all drugs will have different response profiles.
▲ **CRITICAL STEP** Carefully monitor the stability of the prepared solutions before adding to the cultures.

**41|** Perform a new cell viability assay using the seeded 96-well cell culture plate (as prepared in Step 21) based on the cell viability assay procedure (Steps 26–37).

**42|** Repeat the cell viability assays until a dose-response curve is generated for each compound covering its entire range of activity (reaching maximal inhibitory activity).
▲ **CRITICAL STEP** A minimum of two independent experiments (each in triplicate) per data point is recommended. The s.d. of data from independent experiments should not be >5%; otherwise, additional experiments should be performed.
**? TROUBLESHOOTING**
■ **PAUSE POINT** Data can be stored and analyzed at a later time point.

**Drug-dose selection and coding ● TIMING 1 h**
**43|** On the basis of the dose-response curves generated in Steps 38–42, select the number of drug doses and drug concentrations for the required dose efficacy for each compound. Use the dose-response curves generated for each compound in the selection. For an initial screening, test three or more dosage levels (including the dose of zero drug being added) for each compound, as it allows for both the linear contributions (owing to single drug-dose change) and the synergistic contributions (owing to drug-drug interactions) of the drugs to be studied. More dosage levels may be considered if the drugs show a serrated dose-effect curve.
■ **PAUSE POINT** Data can be stored and analyzed at a later time point.

**Initiation of the DE search ● TIMING 5 min**
**44|** Download the MATLAB file called 'RandomGen_Initiation' (**Supplementary Software)** and open it in MATLAB.

**45|** Define the variables *D* (number of drugs in the optimization), *N* (number of drug combinations tested per iteration) and *C* (the number of drug doses tested per drug).
▲ **CRITICAL STEP** In the file provided here, the drug doses will be generated as coded doses (0, 1, 2 and so on). For purposes of the MATLAB code provided, drug doses should always be introduced as coded values.
▲ **CRITICAL STEP** The MATLAB code provided may need to be adapted in the following manner depending on the optimization goal and parameters. These manipulations can be performed in the '.m' file provided in the **Supplementary Software**. The file can be opened in the file editor by double-clicking on the '.m' file from the 'Current Folder' (generally located above the command window). Lines of the code can be deactivated by inserting a '%' at the beginning of each line (nonactive lines of code will appear in green text). First, lines 77–83 should be activated if the optimization goal is to maximize the value of the output response; alternatively, lines 87–93 should be activated if the optimization goal is to minimize the value of the output response (the default setting is for the output value to be maximized). Second, the code is currently written for three coded drug doses; if more or fewer doses are included, the code lines 176–186 must be adapted accordingly. Third, data labels for the results can be adapted in lines 192 and 193.

**46|** Run the MATLAB code (press F5 on the keyboard or Debug and select 'Run RandomGen_Initiation.m').

**47|** Results will appear in the command window. The values of the drug combinations to be tested in the first iteration will appear as a matrix called *P* with the dimension [*D* × *N*], such that each column represents a particular drug combination (1 through *N*) composed of *D* drugs whose doses are indicated by the coded values in each row.
■ **PAUSE POINT** Data can be stored and analyzed at a later time point.

**Preparation of drug combinations ● TIMING 2–4 h**
**48|** Prewarm supplemented cell culture medium at 37 °C for 10 min.

**49|** Prepare and label sterile Eppendorf Safe-Lock tubes.

**50|** Starting from stock solutions prepared in Step 25 or freshly prepared stocks, prepare desired drug concentrations.
▲ **CRITICAL STEP** Each condition and each plate should be properly controlled.

**51|** Perform a new cell viability assay using the seeded 96-well cell culture plates (as prepared in Step 21) based on the cell viability assay procedure (Steps 26–37).

**52|** Determine the cell viability effect for each drug combination and use it as an input for the DE algorithm in Step 56.
▲ **CRITICAL STEP** A minimum of two independent experiments, each in triplicate, per data point is recommended. The s.d. between data from independent experiments should not be >5% (compared between experiments as a percentage of the control wells); otherwise, additional experiments should be performed.
■ **PAUSE POINT** Data can be stored and analyzed at a later time point.

**Implementation of DE algorithm to design combination candidates ● TIMING 1 h**
**53|** Download the MATLAB file called 'DifferentialEvolution' from **Supplementary Software** and open in MATLAB.

**54|** Define the variables *D* (number of drugs in the optimization) and *N* (number of drug combinations tested per iteration).
▲ **CRITICAL STEP** *N* indicates the number of drug combinations to be tested per iteration of the FSC. This value is dependent on the size of the search space, and it can be determined on the basis of the number of dimensions (*D*) in the optimization. In our situation, each drug represents a unique dimension. Studies indicate that values of *N* from 3×*D* to 8×*D* can be effective[42].
▲ **CRITICAL STEP** 'F' and 'c' (**Fig. 2**) are constants, which are set at 0.5. Adapting these constants affect the rate and efficacy of the algorithm's convergence.

**55|** Run the MATLAB code (press F5 on the keyboard or Debug and select 'Run DifferentialEvolution.m').

**56|** Introduce the following data in the command window: *P* (the [*D* × *N*] matrix from the previous iteration, Step 47 in first iteration or Step 57 in later iterations), Output*P* (a [1 × *N*] vector containing the *in vitro* output response corresponding to each of the drug combinations provided in matrix *P*), T (the [*D* × *N*] matrix from the current iteration; this contains only zeros in the first iteration or from Step 57 in later iterations), Output*T* (a [1 × *N*] vector containing the output response corresponding to each of the drug combinations provided in matrix *T*, Step 52).
▲ **CRITICAL STEP** An error comment appears in the command window if the dimension of the data introduced does not match.

**57|** See the results in the command window as two matrices with the dimensions [*D* × *N*], *P* and *T*, where the matrix *P* represents the new population of drug combinations and *T* represents the new trial drug combinations to be tested in the subsequent iteration.

**58|** Repeat Steps 48–57 until the stopping criterion is reached (refer to 'Stochastic search algorithm' in the INTRODUCTION).
▲ **CRITICAL STEP** If the first optimization does not result in the identification of a desirable drug combination, or if it is desired to identify a combination containing fewer compounds (i.e., to eliminate compounds from the optimization), a data modeling step followed by further search refinement steps can be implemented (**Box 1**; see also **Supplementary Tutorial** and **Supplementary Software**).
**? TROUBLESHOOTING**

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 6 | Cells do not detach | Old trypsin solution; cells were not left long enough in solution | Open a fresh trypsin bottle; return the cells to the incubator to allow a longer time for detachment |
| 9 | Cell clumps do not disaggregate | Cells are not fully separated | Mix by gently pipetting |
| 11 | Cells reach full confluency | Cells are growing faster than expected | Passage cells at a lower density |
| 23 | Drug does not fully dissolve in solvent | Stock concentration is too high; incorrect solvent or preparation procedure | Adapt the stock concentration (check with the manufacturer to determine the maximum solubility in solvent) |
| 42 | Results between experiments are not reproducible | Problem with drug stock solutions; cells are not being cultured in a repeatable way | Check all drug stock solutions for precipitants and dates of preparation, and prepare new stock solutions if needed. Monitor both the activity of control cells and the doubling rate; check for cell infections |
| 58 | The algorithm does not optimize (output does not improve over iterations) | Experimental error is too large; original selection of drugs for the optimizations was inappropriate | Repeat experiments; select new drugs |
| **Box 1**, step 4 | Error message appears in the command window | The number of drug combinations and combinations outputs do not match | Run the program again and input data again |

## ● TIMING

Steps 1–11, cell culture and passaging: 20–30 min
Steps 12–21, cell seeding in a 96-well plate: 40 min
Steps 22–25, drug stock and aliquot preparation: 10 min per drug
Steps 26–37, cell viability assay: 2 + 72 h for incubation time per iteration
Steps 38–42, dose-escalation study of individual compounds: 72 h per experiment
Step 43, drug-dose selection and coding: 1 h
Steps 44–47, initiation of the DE search: 5 min
Steps 48–52, preparation of drug combinations: 2–4 h
Steps 53–58, implementation of DE algorithm to design combination candidates: 1 h
**Box 1**, data modeling and analysis: 2 h

## ANTICIPATED RESULTS

The FSC procedure discovers efficient drug combinations for the treatment of disease. The major advantage of the presented method is the large reduction in the required experimental effort as compared with testing all possible combinations. FSC not only allows for the identification of optimal combinations, but it also gives insight into which drugs synergize and which ones display antagonistic activities. This provides information on the working mechanisms of the used drugs. Ultimately, FSC facilitates the selection of mixture components and their dose ratios.

A major finding in all of the experimental cases was that the response surfaces of the drug combinations were smooth near the optimal response[14] (**Fig. 4**). This is considered a benefit, as this means that small changes in the dose of drugs are not expected to result in major changes in the response of the system. This finding is believed to be related to the fact that complex and interconnected biological systems have redundant or compensatory signaling pathways leading to the maintenance of cellular response to external stimuli. Smooth response surfaces near the optimum are expected to have a major impact on translating the *in vitro* results to *in vivo* application. Indeed, the translation of optimal angiostatic drug combinations for the treatment of cancer to application in preclinical models was recently shown to be successful in a mouse tumor model[14]. It is expected that translation into clinical studies in cancer patients is therefore feasible as well.

The regression analysis approach includes second-order terms, allowing for some incorporation of nonlinearity in the system analysis. In our experience, this method has been sufficient to accurately describe cell behavior in response to drug combinations[43]. Moreover, others have also described the contribution of higher-order terms (third order and above) as being minimal (representing <3% of the data variation)[25]. Various other groups have also described the response surface of cells to drug combinations as "highly nonlinear"[28,30] where "sometimes nonlinear curve fitting is desirable or actually required"[44]—for example, the in use of a polynomial fit for the response to a combination of anesthetic drug interactions[45].

An example data set is provided in the **Supplementary Data**. The raw data provided in the first tab of the Excel file represent the results of *in vitro* cell viability inhibition assays performed in the ECRF24 cell line, as described in the protocol above. The drug combinations tested were generated by applying the DE algorithm over multiple iterations while testing four different compounds at the five doses indicated (in **Supplementary Data**, referred to in coded doses as 0–4). The drug combinations and their efficacy are analyzed based on regression analysis using the MATLAB code provided. The results of the regression analysis can be found in the second tab labeled 'Model'. The regression coefficient generated in MATLAB is visualized in a bar graph to facilitate the interpretation of drug contributions. Regression plots for residual analysis are provided (**Supplementary Data**).

It should be noted that in order to limit the search space, our protocol omits the variable timing required for the application of individual drugs. This implies that there may well be, in some cases, a substantial set of possibilities for further future improvement of the best-optimized drug mixtures that we find at present.

Finally, it is expected that the use of low-dose combination therapies—owing to the synergistic effects of drugs and the related reduction of doses—can enhance therapeutic efficacy, while reducing toxic side effects, which may also reduce acquired resistance to the therapy. A future goal of this approach would be the development of personalized treatment approaches, which would be most relevant for the treatment of cancer. The large genetic variation among patients demands the development of patient-tailored medicine. In addition, the rapid genetic drift of tumor cells during the course of therapy can also be addressed by renewed identification of optimal treatment strategies throughout the duration of therapy.



*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

**ACKNOWLEDGMENTS** C.-M.H. acknowledges the support from the Ben Rich-Lockheed Martin Professor Endowment Fund and the Bill and Melinda Gates Foundation. P.N.-S. and A.W.G. acknowledge the support of KWF, the Dutch Cancer Society (Project VU 2014-7234). The authors acknowledge EPFL for financial support. We appreciate A.M. Silva for providing the sketch for **Figure 2**.

**AUTHOR CONTRIBUTIONS** P.N.-S. conceived and designed the protocol, and wrote the manuscript; X.D. designed the protocol, modeled the data, delivered the code and wrote the manuscript; A.W. designed the protocol, delivered the code and wrote the manuscript; C.-M.H. conceived the FSC technology and participated in data modeling, data interpretation and writing of the manuscript; P.J.D. participated in data interpretation and writing of the manuscript; H.v.d.B. initiated the collaboration and participated in data interpretation, and in writing of the manuscript; A.W.G. designed the study, participated in data interpretation and contributed to writing of the manuscript. All authors read and approved the final manuscript.

**COMPETING FINANCIAL INTERESTS** The authors declare competing financial interests: details are available in the online version of the paper.



1. Sawyers, C.L. Cancer: mixing cocktails. *Nature* **449**, 993–996 (2007).
2. De Francesco, R. & Migliaccio, G. Challenges and successes in developing new therapies for hepatitis C. *Nature* **436**, 953–960 (2005).
3. Lehar, J. *et al.* Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nat. Biotechnol.* **27**, 659–666 (2009).
4. Lindsay, M.A. Target discovery. *Nat. Rev. Drug Discov.* **2**, 831–838 (2003).
5. Forrest, S. Genetic algorithms: principles of natural selection applied to computation. *Science* **261**, 872–878 (1993).
6. Bonabeau, E., Dorigo, M. & Theraulaz, G. Inspiration for optimization from social insect behaviour. *Nature* **406**, 39–42 (2000).
7. Al-Shyoukh, I. *et al.* Systematic quantitative characterization of cellular responses induced by multiple signals. *BMC Syst. Biol.* **5**, 88 (2011).
8. Wang, H. *et al.* Mechanism-independent optimization of combinatorial nanodiamond and unmodified drug delivery using a phenotypically driven platform technology. *ACS Nano* **9**, 3332–3344 (2015).
9. Ding, X., Xu, H., Hopper, C., Yang, J. & Ho, C.M. Use of fractional factorial designs in antiviral drug studies. *Qual. Reliab. Eng. Int.* **29**, 229–304 (2013).
10. Honda, Y. *et al.* Guiding the osteogenic fate of mouse and human mesenchymal stem cells through feedback system control. *Sci. Rep.* **3**, 3420 (2013).
11. Sun, C.P. *et al.* Integrative systems control approach for reactivating Kaposi's sarcoma-associated herpesvirus (KSHV) with combinatory drugs. *Integr. Biol. (Camb)* **1**, 123–130 (2009).
12. Tsutsui, H. *et al.* An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells. *Nat. Commun.* **2**, 167 (2011).
13. Weiss, A. *et al.* A streamlined search technology for identification of synergistic drug combinations. *Sci. Rep.* **5**, 14508 (2015).
14. Weiss, A. *et al.* Rapid stochastic optimization of drug combination for inhibiting angiogenesis in cancer growth. *Angiogenesis* **9462**, 233–244 (2015).
15. Wong, P.K. *et al.* Closed-loop control of cellular functions using combinatory drugs guided by a stochastic search algorithm. *Proc. Natl. Acad. Sci. USA* **105**, 5105–5110 (2008).
16. Yoon, B.J. Enhanced stochastic optimization algorithm for finding effective multi-target therapeutics. *BMC Bioinformatics* **12** (suppl. 1): S18 (2011).
17. Yu, F. *et al.* Control of Kaposi's sarcoma-associated herpesvirus reactivation induced by multiple signals. *PLoS ONE* **6**, e20998 (2011).
18. Ding, X. *et al.* Cascade search for HSV-1 combinatorial drugs with high antiviral efficacy and low toxicity. *Int. J. Nanomed.* **7**, 2281–2292 (2012).
19. Yu, H. *et al.* Optimizing combinations of flavonoids deriving from astragali radix in activating the regulatory element of erythropoietin by a feedback system control scheme. *Evid. Based Complement. Altern. Med.* **2013**, 541436 (2013).
20. Storn, R.M. & Price, K.V. Differential evolution—a simple and efficient heuristic for global optimization over continuous spaces. *J. Global Optim.* **11**, 341–359 (1997).
21. Das, S. & Suganthan, P.N. Differential evolution: a survey of the state-of-the-art. *IEEE Trans. Evol. Comput.* **15**, 4–31 (2011).

22. Chakraborty, U.K. *Advances in Differential Evolution* (Springer Verlag, 2008).
23. Yoon, H. *et al.* Modeling to predict growth/no growth boundaries and kinetic behavior of *Salmonella* on cutting board surfaces. *J. Food Prot.* **75**, 2116–2121 (2012).
24. Van der Borght, K. *et al.* Cross-validated stepwise regression for identification of novel non-nucleoside reverse transcriptase inhibitor resistance associated mutations. *BMC Bioinformatics* **12**, 386 (2011).
25. Wood, K., Nishida, S., Sontag, E.D. & Cluzel, P. Mechanism-independent method for predicting response to multidrug combinations in bacteria. *Proc. Natl. Acad. Sci. USA* **109**, 12254–12259 (2012).
26. Seber, G.A.F. & Lee, A.J. *Linear Regression Analysis* 2nd edn (Wiley, 2003).
27. Chou, T.C. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* **70**, 440–446 (2010).
28. Feala, J.D. *et al.* Systems approaches and algorithms for discovery of combinatorial therapies. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **2**, 181–193 (2010).
29. Dancey, J.E. & Chen, H.X. Strategies for optimizing combinations of molecularly targeted anticancer agents. *Nat. Rev. Drug Discov.* **5**, 649–659 (2006).
30. Park, M., Nassar, M. & Vikalo, H. Bayesian active learning for drug combinations. *IEEE Trans. Biomed. Eng.* **60**, 3248–3255 (2013).
31. Decker, S. & Sausville, E.A. Preclinical modeling of combination treatments: fantasy or requirement? *Ann. NY Acad. Sci.* **1059**, 61–69 (2005).
32. Tol, J. *et al.* Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. *N. Engl. J. Med.* **360**, 563–572 (2009).
33. Jia, J. *et al.* Mechanisms of drug combinations: interaction and network perspectives. *Nat. Rev. Drug Discov.* **8**, 111–128 (2009).
34. Calzolari, D. *et al.* Search algorithms as a framework for the optimization of drug combinations. *PLoS Comput. Biol.* **4**, e1000249 (2008).
35. Zimmermann, G.R., Lehar, J. & Keith, C.T. Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discov. Today* **12**, 34–42 (2007).
36. Borisy, A.A. *et al.* Systematic discovery of multicomponent therapeutics. *Proc. Natl. Acad. Sci. USA* **100**, 7977–7982 (2003).
37. Lamb, J. *et al.* The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science* **313**, 1929–1935 (2006).
38. Potti, A. *et al.* Genomic signatures to guide the use of chemotherapeutics. *Nat. Med.* **12**, 1294–1300 (2006).
39. Lehar, J. *et al.* Chemical combination effects predict connectivity in biological systems. *Mol. Syst. Biol.* **3**, 80 (2007).
40. Nelander, S. *et al.* Models from experiments: combinatorial drug perturbations of cancer cells. *Mol. Syst. Biol.* **4**, 216 (2008).
41. Zhao, B., Hemann, M.T. & Lauffenburger, D.A. Intratumor heterogeneity alters most effective drugs in designed combinations. *Proc. Natl. Acad. Sci. USA* **111**, 10773–10778 (2014).
42. Gamperle, R.M., Sibylle, D. & Koumoutsakos, P. A Parameter Study for Differential Evolution. in *Advances in Intelligent Systems, Fuzzy Systems, Evolutionary Computation* (eds. Grmela, N. & Mastorakis, A.) 293–298 (WSEAS Press, 2002).
43. Ding, X. *et al.* Discovery of a low-order drug-cell response surface for applications in personalized medicine. *Phys. Biol.* **11**, 065003 (2014).
44. Tallarida, R.J. Drug synergism: its detection and applications. *J. Pharmacol. Exp. Ther.* **298**, 865–872 (2001).
45. Minto, C.F. *et al.* Response surface model for anesthetic drug interactions. *Anesthesiology* **92**, 1603–1616 (2000).
46. Price, K.V., Storn, R.M. & Lampinen, J.A. *Differential Evolution: A Practical Approach to Global Optimization* (Springer, 2005).
47. Storn, R.M. *Differential Evolution (DE)* http://www1.icsi.berkeley.edu/~storn/code.html.