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RESEARCH ARTICLE

Optimization of muscle cell culture media using nonlinear design of experiments

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Abstract

Optimizing media for biological processes, such as those used in tissue engineering and cultivated meat production, is difficult due to the extensive experimentation required, number of media components, nonlinear and interactive responses, and the number of conflicting design objectives. Here we demonstrate the capacity of a nonlinear design-of-experiments (DOE) method to predict optimal media conditions in fewer experiments than a traditional DOE. The approach is based on a hybridization of a coordinate search for local optimization with dynamically adjusted search spaces and a global search method utilizing a truncated genetic algorithm using radial basis functions to store and model prior knowledge. Using this method, we were able to reduce the cost of muscle cell proliferation media while maintaining cell growth 48 h after seeding using 30 common components of typical commercial growth medium in fewer experiments than a traditional DOE (70 vs. 103). While we clearly demonstrated that the experimental optimization algorithm significantly outperforms conventional DOE, due to the choice of a 48 h growth assay weighted by medium cost as an objective function, these findings were limited to performance at a single passage, and did not generalize to growth over multiple passages. This underscores the importance of choosing objective functions that align well with process goals.

KEYWORDS
cultured meat, doe, dycors, machine learning, media optimization

1 | INTRODUCTION

Cell culture media is a critical component of bioprocesses such as pharmaceutical manufacturing and the emerging field of cultivated meat products. Optimizing culture media is a difficult task due to the extensive experiments required, number of media components, nonlinear and interactive responses from each component, and conflicting design objectives. Additionally, for cultured meat products, media needs to be less expensive than those currently deployed for other cell culture processes (e.g. biopharmaceutical production), food-grade, consider safety, component stability, and effects on sensory characteristics of final products. Without much in the way of first principles models for these objectives, especially for adherent mammalian muscle cells used for cultivated meat production (as well as fat and connective tissues), media optimization must be done experimentally with constraints on inputs, outputs, and number of experiments.

Optimizing one factor at a time or with random experiments is still the most common way of exploring design space. This strategy is very inefficient for large systems (culture media such as DMEM may have up to 30 components [1]) and is unable to consider interactions among media components. Design-of-Experiments (DOE) methods are better able to manage large numbers of components in fewer experiments using Factorial, Fractional Factorial, Plackett-Burman, and Central Composite Designs where linear and polynomial models can correlate first order and interactive effects of media components. In general,

Abbreviations: AB, AlamarBlue; DMEM, Dulbecco's modified eagle medium; DOE, design-of-experiments; FBS, fetal bovine serum; GM, growth/generic media; HND, hybrid nonlinear designer; RBF, radial basis function

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DOE methods are able to optimize < 10 variables,[2] and with the help of screening designs can solve problems > 25 variables,[3] though at the expense of ignoring interactions, screened variables, and easily costing > 100 experiments (when combining typical screening and factorial experiments, although this number can be quite lower if < 5 variables are explored). Experimental optimization of media has also been done using stochastic methods such as genetic algorithms,[4] and this approach is generally suited to optimizing systems of dimensionality > 15 where DOE methods can become experimentally cumbersome, but also take ~ 200 experiments.

Because the size of the design space increases exponentially with the number of design variables, a natural advance was to use response surface models to capture information about interactions and nonlinearity. These techniques can then be used to sequentially identify optimal culture conditions while simultaneously improving modeling accuracy. Oftentimes experimenters will employ polynomial models to find optimal culture conditions[5] but only after extensive DOE to reduce the dimensionality of the problem space to < 5. More advanced modeling techniques are neural networks, decision trees[6] and Gaussian processes,[7] which are often better at generalizing noisy, nonlinear, and multi-modal data. When combined with global optimization methods, Zhang and Block demonstrated that these response surface methods can optimize problems with > 20 variables in less than half the number of experiments as traditional DOE.[8] Recently Cosenza and Block[9] further improved the robustness of this algorithm by using a hybrid optimization scheme validated on simulated design problems.

Here we employ this novel nonlinear experimental design algorithm called HND to optimize the proliferation of C2C12 cells while simultaneously reducing media cost by modeling the response surface of culture conditions using an RBF with a hybridized global/local optimization scheme. We then compare this approach to a more traditional DOE method. The organization of this article is as follows: Section 2 includes an outline of the experimental and computational methods use in media optimization, Section 3 goes over the results and Section 4 details a discussion of the results and current challenges.

2 | MATERIALS AND METHODS

2.1 | Media components and cell line

Table 1 lists the 30 components of the media system, concentration ranges, and the concentration of the control growth media (GM) used in this work. GM is based on a formulation of DMEM + 10% FBS from HiMedia Cell Culture with 4.5 g Glucose L^{-1} and L-Glutamine where FBS is fetal bovine serum (Biowest). All components were stored as aqueous stock solutions in 2–6°C sterilized using 0.2 μm pore size micro-filtration (Pall Corporation Acrodisc). The pH was adjusted to 7.2 using 1 M HCl or NaOH solution, and Sodium Bicarbonate (Sigma) buffer at 1850 mg L^{-1} was added.

C2C12 muscle cells were used for all experiments (ATC). The cells were stored in liquid N_2 in 10% DMSO (Sigma), 20% FBS, 70% GM at passage 15. To generate enough cells for these experiments, cells were taken out of storage, thawed, centrifuged at 1500 g for 5 min and re-suspended in DMEM (Glibco) + 10% FBS in 15 cm cell culture plates (Cellstar, Greiner Bio-One). Cells were then trypsinized (Gen-Clone) in their log phase of growth (~ 50% confluence, or about two days of growth) and plated on 96 well plates (Cellstar, Greiner Bio-One). To plate the cells, trypsinized cells are suspended in phosphate buffered solution (PBS Glibco) and counted using a hemocytometer. The PBS volume was then adjusted so that 5000 cells per well (~ 15,625 cells cm^{-2}) could be seeded using 50 μL of PBS into 150 μL of the media being tested (total well volume of 200 μL). The cells were incubated at 37°C and 5% CO_2 for 48 h post-seeding before measurements of proliferation were made with replicates. For six well plate experiments (Cellstar, Greiner Bio-One) a total volume of 3 mL was used with the same ratios of PBS to media and seeding density (150,000 cells per well), with all other steps being the same.

2.2 | Assays and objective function

After 48 h of incubation, the performance of the media was measured using AlamarBlue[10] metabolic colorimetric assay (AB). After pipetting in 10% volume of AB assay (20 μL) for each well, all wells were left to incubate for 3 h at 25°C and 5% CO_2. The %AB reduction was measured using a microplate reader at 600 and 570 μm using Equation (1) with six replicates of each experimental and control well.

$$\%AB = \frac{117216 * \lambda_{570,media} - 80586 * \lambda_{600,media}}{155677 * \lambda_{600,control} - 14625 * \lambda_{570,control}} \quad (1)$$

To quantify the relative proliferation of cells after 48 h of growth, the ratio of %AB for a given medium to %AB for basic GM was used as a metric of the success. The economic cost of a medium was considered by normalizing the %AB ratio by the volume of FBS, which constitutes the vast majority of the media cost.[11] Therefore, the objective function α and the optimization problem used in this work (finding the best media components X^*) are as follows, where $\bar{X}_{FBS}$ is the normalized volume of FBS ranging from [0, 1].

$$X^* = argmax_X \alpha(X)$$

$$\alpha(X) = \frac{\%AB/\%AB_{GM}}{1 + \bar{X}_{FBS}}$$

$$\bar{X}_i = \frac{X_i - X_{i,low}}{X_{i,high} - X_{i,low}}$$

This objective function strikes a balance between a proportionality to cell proliferation and cost, and ease of use. A more elaborate objective function that describes multi-passage dynamics or further economic costs could be employed, but at the expense of significantly more time and labor.

TABLE 1 Details of media design space | components and bounds used in media optimization for proposed method (HND), control optimization method (DOE), and commercial (GM) indicated

		Concentration [mg L^{-1}]				
Component		**GM**	**Low**	**High**	**HND**	**DOE**
Calcium chloride	EMD	265	132.5	530	287.3	265
Ferric nitrate	Fischer	0.1	0.05	0.2	0.1	0.1
Magnesium sulphate	RPI	97.7	48.85	195.4	176.8	97.7
Potassium chloride	Fischer	400	200	800	555.8	200
Sodium chloride	Fischer	6400	3200	12800	8182.8	6400
Glycine	Fischer	30	15	60	23.1	30
L-Arginine	Spectrum	84	42	168	76.1	84
L-Cystine	RPI	62.6	31.3	125.2	94.7	62.6
L-Glutamine	Spectrum	584	292	1168	977.8	584
L-Histidine	Spectrum	42	21	84	75.6	42
L-Isoleucine	Acros	105	52.5	210	125.8	105
L-Leucine	Acros	105	52.5	210	92.1	105
L-Lysine	RPI	146	73	292	207.5	146
L-Methionine	Spectrum	30	15	60	45.5	30
L-Phenylalanine	AMRESCO	66	33	132	87.6	66
L-Serine	AMRESCO	42	21	84	52.6	42
L-Threonine	Spectrum	95	47.5	190	146.4	95
L-Tryptophan	Biosynth	16	8	32	24.9	16
L-Tyrosine Disodium Salt	RPI	103.8	51.9	207.6	152.3	104
L-Valine	Spectrum	94	47	188	117	94
Choline chloride	Sigma	4	2	8	4.5	4
D-Ca-Pantothenate	Acros	4	2	8	5.7	4
Folic acid	TCI	4	2	8	5.2	4
Nicotinamide	Sigma	4	2	8	6.8	4
Pyridoxal hydrochloride	Acros	4	2	8	3.7	4
Riboflavin	Sigma	0.4	0.2	0.8	0.5	0.4
Thiamine hydrochloride	Sigma	4	2	8	4	4
I-Inositol	Fischer	7.2	3.6	14.4	6.4	7.2
D-Glucose	Sigma	4500	2250	9000	6145.7	9000
FBS	Biowest	10%	5%	20%	6.8%	5%

2.3 | Experimental design algorithm

A novel hybrid nonlinear experimental design algorithm (HND) was developed [9] to optimize high dimensional experimental design systems such as the one outlined above. It is based on a truncated genetic algorithm (TGA) method [8] hybridized with a dynamic coordinate search framework (DYCORS).[12] This method starts by constructing an RBF approximation $\hat{y}$ of the system from an initial set of experiments with inputs and outputs $\{X_0,\alpha_0\}$ respectively. The RBF takes the form of a sum of n_c cluster λ_i-weighted radial functions $\varphi(x, x')$ in Equation (2).

$$\hat{y} = \sum_{i=1}^{n_c} \lambda_i \phi(r_i) \tag{2}$$

The radial functions project a set of [0, 1] normalized inputs x and x' (in this case two media concentrations) into a single output space using the Euclidean distance $r = ||x - x'||_2^2$. This quantifies the difference between two media combinations for arbitrary media components. Two media that are more similar have smaller r values, so are going to have similar predictions of $\hat{y}$. The radial function used in this work was the cubic function $\varphi(x, x') = r^3$. The weights are determined by solving the linear equation for $\Phi(X, X)$ for a training set of data that has been collected $\{X, \alpha\}$.

$$\lambda = \left(\Phi\Phi^T\right)^{-1}\Phi^T\alpha \tag{3}$$

To find the optimal location of RBF nodes n_c we used the K-Means Clustering Algorithm. This algorithm was repeated for $K = 4$

Data: ;
Initial Data $\{X_0, Y_0\}$;
Max Batches B;
Objective $\alpha(x)$
Result: Optimal Design X^*
for $b = 1 : B$ **do**
  Get RBF Approximation;
  $\alpha(x) \approx \hat{y}(x) = \Sigma_{i=1}^{n_c} \lambda_i \phi(r_i)$;
  Run HND Algorithm;
  $X_{TGA} = argmax_{TGA}\, \hat{y}(x)$;
  $X_{DYCORS} = argmax_{DYCORS}\, \hat{y}(x)$;
  $X_b = X_{TGA} \cup X_{DYCORS}$;
  Conduct New Experiments;
  $Y_b = \alpha(X_b)$;
end
$X^* = argmax\, \alpha(X)$

FIGURE 1 Hybrid nonlinear design (HND) algorithm

cross-validated data splits for each batch of experiments, where the n_c with the lowest cross-validated error for the given training set was chosen as the optimal number of clusters. Cross-validation is critical for making sure models generalize well for small amounts of noisy data. In general, higher n_c makes the model more complex (wiggly), so here we balance accuracy with model simplicity/generalizability.

Using the trained RBF model, the two arms of our algorithm, TGA, and DYCORS, each suggest five experimental conditions for a total of 10 experiments per batch within the design space [× 1/2, × 2] of the GM (see Table 1) that optimize α. The TGA arm runs a genetic algorithm (a stochastic global optimization method) over the RBF model to predict the best designs. Because the model is based on a small amount of noisy data, the genetic algorithm is stopped before it can converge to implicitly consider model and experimental uncertainty. The DYCORS arm of the algorithm searches in the region around the best design and picks the best predicted set of designs in that region, which expands and contracts based on the quality of previous experiments. The new experiments are conducted and the resulting data is used to correct and retrain the RBF model. To allow the RBF model to generalize better during early periods of optimization, 30 randomly selected experimental conditions were taken initially. The optimization loop was stopped when the α quality of the media showed a lack of improvement. The general framework for the HND is shown in Figure 1.

As a control method, a traditional DOE was used to optimize the same media design problem in three steps. (i) A 'Leave-One-Out' (LOO) experiment was conducted where a media composed of all components at their GM concentrations, excluding each individual component, were tested for their proliferation capacity using the %AB metric (α was not used because all media had the same amount of FBS), similar to what was done in previous work.[13] The lowest performing components had their concentrations fixed at their respective GM concentrations. Next (ii) a Folded/Un-Folded Plackett-Burman design was implemented with the remaining components at the upper and lower bounds of the design problem. This was done to determine the first order linear effects of each component on the objective function α. A linear model to predict α was used in conjunction with a LASSO algorithm (Hastie, 2017, p. 68) to rank the most important first order effects, and all but the highest impact components were kept at their GM concentrations. Finally, (iii) the remaining components were used to design a Central Composite Design (CCD) where experiments are spread out across the design space to more thoroughly explore potential optimal designs. The best α design from this DOE method was considered the optimal DOE design.

2.4 | Computing environment

Hardware used: Dell Precision 5820 Tower, Intel Xeon W-2145 DDR4-2666 Processor (3.7 GHz), 32 GB Memory. Software used: MATLAB R2019a with Bioinformatics Package.

3 | RESULTS

3.1 | Performance of traditional DOE for media optimization

The DOE-LOO step identified Ferric Nitrate, $MgSO_4$, Glycine, L-Isoleucine, Choline Chloride, Riboflavin, and Thiamine HCl as components that, when left out of GM, had no (or positive) statistical effect on %AB after 48 h post-seeding (30 experiments needed). These components were set to their respective GM concentration for all subsequent DOE experiments. Next, the DOE-PB with LASSO identified the six most α-important components of the remaining 23 components (KCl, L-Glutamine, Glucose, FBS, L-Cystine, L-Serine). To reduce the number of experiments for the DOE-CCD design, L-Cystine and L-Serine were kept constant at × 1/2 normalized units above and below their GM midpoint concentrations respectively (10.4 and 28 mg mL^{-1}) based on the sign of their coefficients (48 experiments required). The remaining four components in the CCD had their upper/lower bounds changed to × 1/2 normalized units above (KCl, L-Glutamine, FBS) and below (Glucose) their GM midpoints. The remaining components were varied in a CCD design, with the best medium being 200 mg L^{-1} KCl, 388 mg L^{-1} L-Glutamine, 9000 mg L^{-1} Glucose, 5% FBS (25 experiments) shown in detail in Table 1. An 80% increase in α at 48 h post-seeding over GM was measured (Figure 2 left) using 50% less FBS than GM.

3.2 | Performance of novel HND for media optimization

For the HND optimization loop, α was used as the objective function and calculated using %AB measured at 48 h post-seeding at 96 well plate scale (the exact same as the DOE method). The RBF was initially trained with 30 randomly selected experiments. Figure 2 shows that the average HND designs improved in both α and %AB metric over time (both cost and proliferation) quickly overcoming standard GM and achieving similar results to the best DOE design (an α difference of 13.3%) with 70 experiments. We have included the proliferation metric

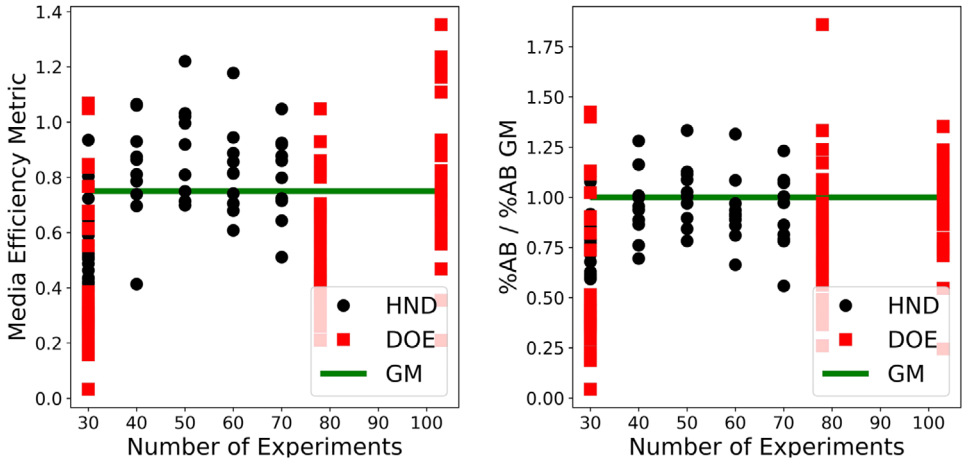

FIGURE 2 Iterative improvement of media using HND and DOE | (left) media efficiency metric (right) %AB Proliferation. Both HND and DOE improve over GM

(%AB / %AB GM) in Figure 2 for completeness even though it was not used as the objective function α in this work. The HND was stopped at 70 experiments because both %AB and α stopped improving. The best medium found had an α measured to be 56% better than GM during the optimization loop using 32.5% less FBS than GM.

3.3 | Comparison of media resulting from novel HND and traditional DOE

Figure 3 shows the differences between the optimal media. For the most part the HND identified optimal concentrations that were slightly elevated compared to DOE, except for KCl, FBS, and Glucose. It is also notable that both HND and DOE determined that Glucose and FBS should be elevated and reduced in relative to GM. Figure 4 shows the media efficiency metric α plotted against the component concentrations for all experiments, demonstrating the nonlinear, interactive, and ultimately non-trivial nature of this experimental design optimization problem. These α optimal HND and DOE designs were then tested against GM using %AB at 24, 48, and 72 h post-seeding (Figure 5), where the designed media have high %AB relative to GM but that advantage is reduced over time. As a further check, α was calculated using raw cell number normalized by the volume of FBS in each experiment (at six well plate scale) where it was found HND and DOE again outperformed GM (Figure 5) in terms of the objective function α. However, both HND and DOE produced 8% and 9% fewer cells respectively, using 70 and 103 total experiments respectively. This higher α comes from their lower levels of FBS.

3.4 | Evaluation of optimized media in multi-passage proliferation

Finally, the C2C12 cells were grown in optimal HND, DOE, and GM across five passages to mimic an industrial process where multi-passage dynamics could have large effects on media design. Figure 6 indicates GM cumulatively grew more cells than HND and DOE optimal media by the second passage, and by the third passage had done so at higher α (again, approximated by number of cells normalized by volume of FBS). Both the optimal HND and DOE media performed roughly the same in terms of cumulative number of cells and media efficiency, but with 9x and 11x fewer cells than GM respectively and without a proportional decrease in cost per cell.

4 | DISCUSSION

It is notable that, despite 30 components used, the HND was able to design a similar media to DOE with a similar degree of proliferation %AB and α in fewer experiments. Additionally, this DOE was more efficient than any single DOE, suggesting that the HND is much more efficient and simpler to use than the typical approach to high dimensional optimization. This is valuable in optimizing media due to the difficulty in collecting large amounts of data with many components. The reasons for the success of this method are likely (i) the balance between global and local optimization, and (ii) the ability of the HBD to accumulate information using the RBF, which can regress on nonlinear, noisy, and interaction-heavy problems, reducing the need for cumbersome dimensionality-reduction experiments used in the traditional DOE.

For the most part HND suggested higher concentrations of most media components than GM or DOE, except for KCl, FBS, and Glucose. This is likely because the DOE method utilized dimensionality reduction. That is, factors that demonstrated insignificant effects were fixed at their GM level and no longer included in the optimization. On the other hand, HND could vary components throughout the optimization process, including increasing component concentrations when they had even a small positive effect. Inclusion of a per component cost (rather than just the cost of FBS) might dampen this effect.

While the RBF can model nonlinear and interactive processes, the effect of each component on α is unclear without further experiments

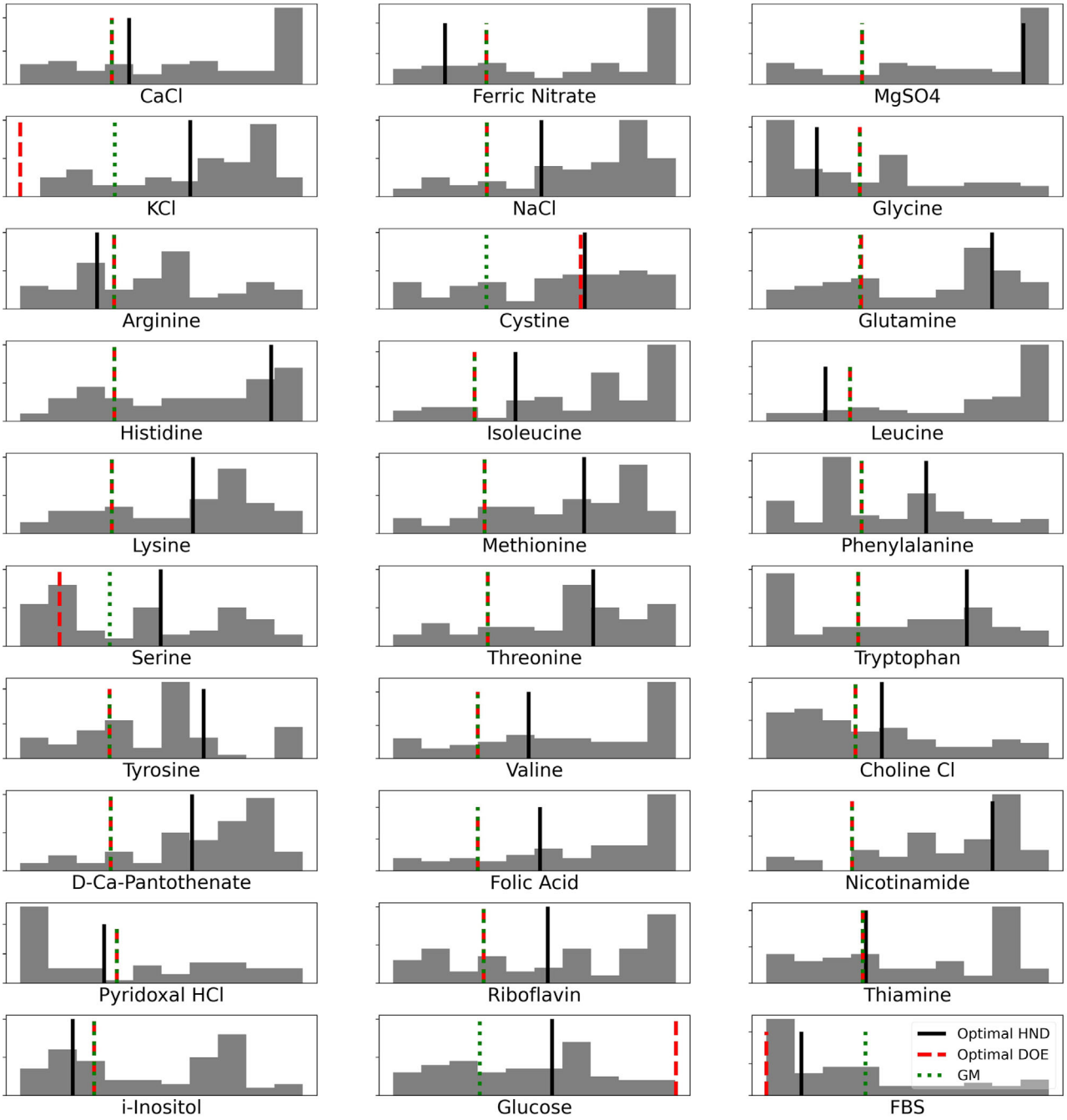

FIGURE 3 Distribution of components generated by HND | histogram of HND chosen component concentrations from low to high bound, best DOE and HND results also compared to GM (as horizontal lines and in Table 1)

or model validation, a disadvantage of the HND approach. Nonetheless, sensitivity analysis using VARS[14] was conducted and indicates FBS, Glucose, and $MgSO_4$ likely have a significant effect on α, while other effects are more difficult to determine with the limited data available. Sobal sensitivity analysis utilizing polynomial regression likewise determined FBS, $MgSO_4$, and L-Phenylalanine were the most explanatory components when taking component-component interactions into account. Focusing on optimizing only those components might bring further improvements, which is now feasible because fewer experiments were needed to arrive at this conclusion. Another issue was that the HND algorithm often did not change experimental conditions enough, leading to heavy clustering around early high performing local optima (as seen in Figure 3 and 4). Myopia (shortermism) should be encoded into the DYCORS arm of the HND to allow for more exploration of the design space, while balancing the need for exploitation of regions of the design space that show promise. It is also possible that initializing the optimization with a more dispersed design would yield a more successful optimization. However, results from Zhang and Block[15] indicate that the initialization strategy used may not have a large effect. In reality, the impact of initialization is likely

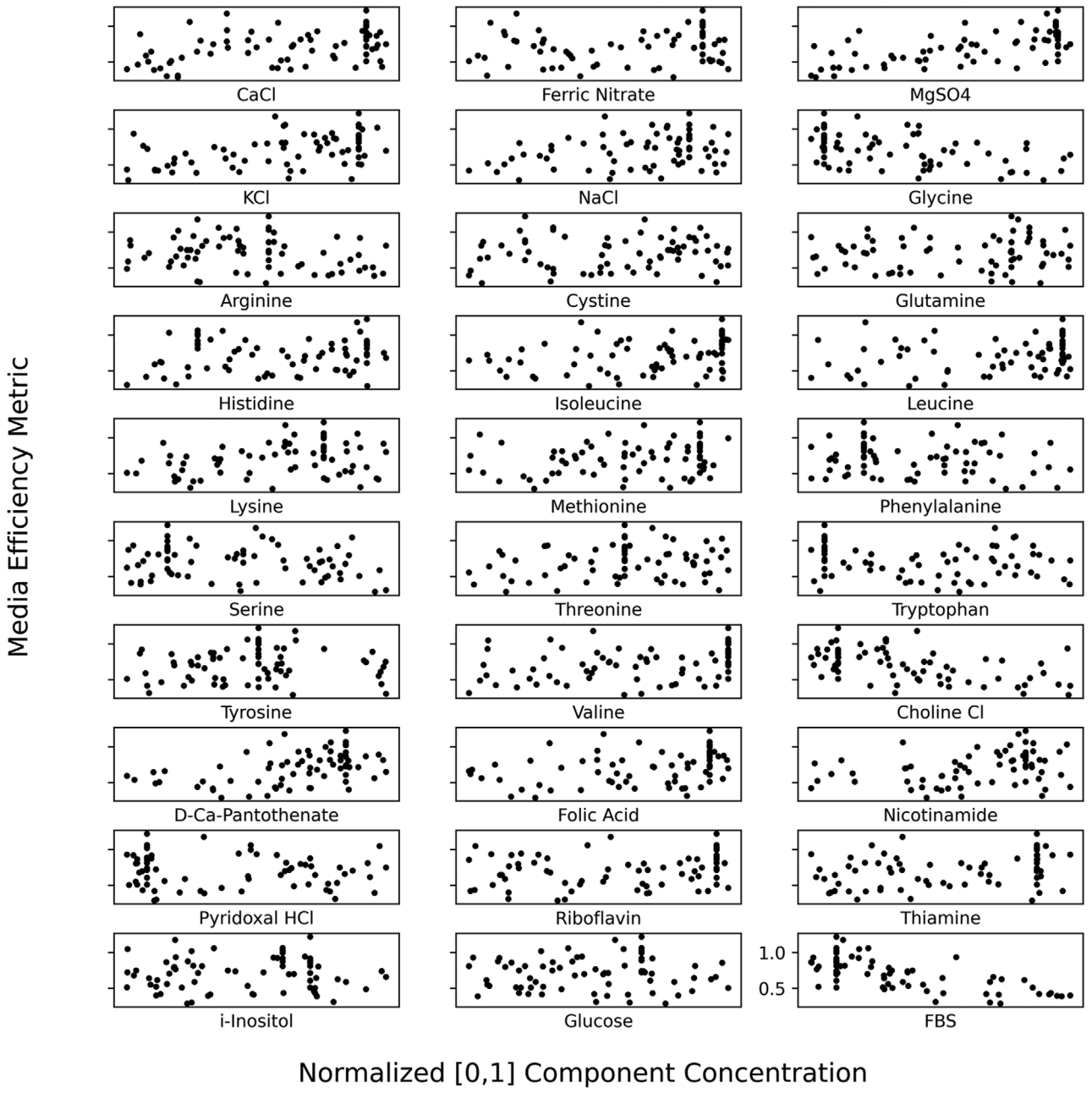

FIGURE 4 Input and output of media generated by HND | each dot represents an experiment designed by HND at a chosen component concentrations (normalized to be 0 to 1) and the respective media efficiency metric α

to be a strong function of the design surface and how close initial points are to the true optimum, neither of which are known a priori.

Using α as a metric, HND performs similar to DOE, and both better than GM (Figure 2). This is true over multiple days after cell seeding and is true when using cell number to calculate α (Figure 5), seemingly validating the use of %AB at 48 h post-seeding in approximating proliferation more generally. However, when measuring cell number at multiple passages (Figure 6) both designed media perform worse than GM. This is because the objective function α relied on measurements without multiple passages, so does not account for the dynamics of long-term cellular growth. This was a major shortcoming of the objective function picked, but not the HND or DOE itself. Future work in media design should incorporate more relevant metrics for optimization, such as a multi-passage objective function. Additionally, the %AB metric was not a perfect measure of cell number. Figure 5 (left) and Figure 2 appears to indicate HND and DOE media outperform GM, but when cell number is measured both optimal media have 8–9% fewer cells. Because Alamar-Blue is a metabolic indicator, using it in the objective function for both methods may have biased the process towards higher metabolic activity rather than more proliferation.

Despite these shortcomings, the HND has been demonstrated to be able to optimize high dimensional experimental systems. In our

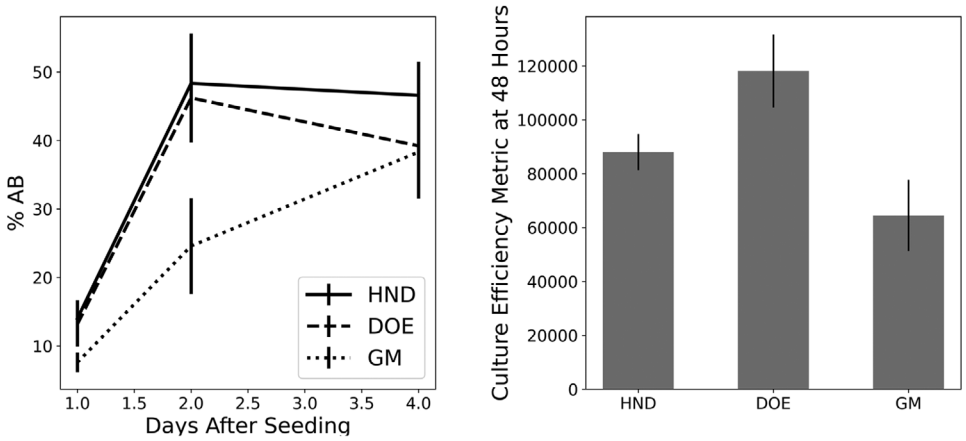

FIGURE 5 Result of optimal HND and DOE experiments | (left) %AB Proliferation over time in 96 well plates, error bars are standard deviation of six replicates, seeded at 5000 cells per well (right) cell efficiency metric at 48 h post-seeding in six well plates, error bars are standard deviations of three replicates, seeded at 150,000 cells per well. The media efficiency metric was approximated here by dividing number of cells by concentration of FBS. Raw cell number for HND, DOE, and GM were 594,000, 590,000, and 640,000 cells per well respectively

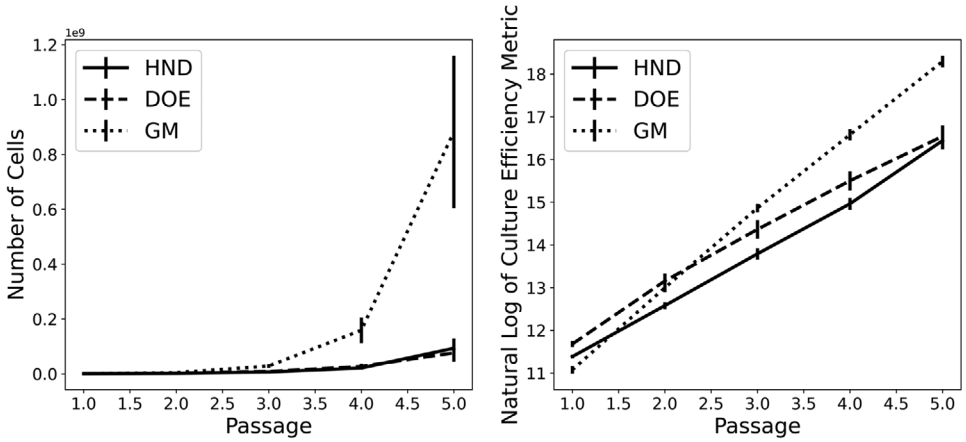

FIGURE 6 Optimal media over multiple passages | these media were the best found in optimization experiments. All cell numbers were taken at 48 h post-seeding using a hemocytometer in six-well plates, error bars are standard deviations of three replicates, seeded at 150,000 cells per well (left) (right) natural log of approximate efficiency of media. The media efficiency metric was approximated here by dividing number of cells by concentration of FBS

previous work in media optimization, fewer variables (21 components) required more experiments (73–94 data points) to complete. In this work, we demonstrate optimization of 30 components with 70 experiments with no dimensionality reduction or screening designs, to our knowledge, a unique accomplishment in experimental optimization efficiency. Therefore, this represents a valuable proof of concept in the field of experimental optimization. While not able to fully replace first principles understanding of systems often based on the DOE approach (which is ill-advisable in any case), we show that the HND could aid in the optimization of the hardest design problems, including those found in the bioprocessing and larger cultivated meat industry, reducing the cost of experimentation and time-to-market for a new product.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

AUTHOR CONTRIBUTIONS

Z. C.: Investigation; methodology; software; writing-original draft. K. B.: Resources; supervision; validation; writing-review & editing

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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