Equivalent Change Enrichment Analysis: Assessing Equivalent and Inverse Change in Genes and Biological Pathways between Diverse Experiments

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Abstract

In silico functional genomics have become a driving force in the way we interpret and use gene expression data, enabling researchers to understand which biological pathways or molecular functions are likely to be affected by the treatments or conditions being studied. There are many approaches, but a number of popular methods determine if a set of modified genes has a higher than expected overlap with genes known to function as part of a pathway (functional enrichment testing). Recently, researchers have started to apply such analyses in a new way: to ask if the data they are collecting show similar disruptions to biological functions as some reference data. Examples include studying whether or not similar genes are perturbed in smokers vs. users of e-cigarettes, or whether a new mouse model of schizophrenia is justified, based on its similarity in cytokine expression to a previously published model. However, there is a dearth of robust statistical methods for testing hypotheses related to these questions. This work proposes a novel statistical approach to testing if the observed perturbances in two biological datasets cause equivalent biological functional changes and shows that it can provide robust and meaningful results.

Introduction

In silico functional genomics have become a standard approach in enabling researchers to use transcriptomics to understand biological pathways or molecular functions affected by the treatments or conditions they are studying. There are many approaches, but a number of popular methods determine if a set of modified genes has a higher than expected overlap with genes known to function as part of a pathway (functional enrichment testing)[1-3]. Increasingly, researchers are asking a different version of this question: i.e., they want to know if the data they are collecting show similar functional disruptions as some reference data. For example, Shen, et al. studied whether similar genes were perturbed in smokers vs. users of e-cigarettes[4]. Gil-Pisa, et al. justified the use of their mouse model of schizophrenia based on its similarity in cytokine expression to a previously published model[5]. Martins-de-Souza, et al. showed that responders showed the same pathways were affected, but in opposite directions, in poor vs. good responders to anti-psychotics[6]. Clearly, this idea has many potential applications. Unfortunately, we currently lack statistically sound approaches for most such analyses.

Currently, most researchers are using a relatively straightforward approach. They simply assess whether or not a large number of the same genes in some relevant pathway are up and down regulated in the same or different directions[7,8]. However, frequently there is no attempt to determine the probability of this occurring by chance, bringing the interpretability and reproducibility of such results into question. In the special case for which data are drawn from the same experiment, there has been work done on equivalence testing for gene expression data[9,10], which would allow one to study some of these questions.

For drug-repurposing, Connectivity Mapping was introduced to address this need. This approach assesses the correlation in ranked lists of genes, with the intent of identifying gene profiles for drugs that are correlated, or anti-correlated to a researcher's own gene signature[11], but this approach is not designed to identify specific biological functions that are similar across experiments. The same is true of other methods, such as openSesame[12] or the extreme cosine method (XCos)[13], which were developed later. Even for drug repurposing, this may be an important point. That is, it may be important to be similar only in terms of certain pathways but not others. Therefore, a method that can systematically identify pathways with similar (or inverted) perturbations could be of great use. Furthermore, it would be very useful to be able to test at the level of individual genes and not just gene signatures, because in some cases this may be more critical, but it is unaddressed by current methods.

In this work, we propose a set of methods that seek to accomplish all these goals, based on equivalence testing at the individual gene level, and a new functional genomics approach. There are many potential benefits, including the ability to focus on genes that may be more directly relevant to the experimental question, drug screening for treatments that have similar effects, demonstrating the viability of a new mouse model, and many other potential uses.

Equivalence Testing

Historically, most of the focus of hypothesis testing in statistics has been on establishing a difference. For example, an experiment might seek to discover if one treatment outperformed another. However, many important questions center on the question of equivalence. For example, one might seek to learn if one treatment is "as good" as another. Typical hypothesis tests of mean difference should not be applied in this scenario for a number of reasons, including: i) a failed hypothesis test leading a researcher to conclude equivalence would result in equivalence conclusions whenever there is simply low precision[14], and ii) a successful hypothesis test can lead a researcher to conclude there is a difference, when the magnitude of the difference is not meaningful due to high precision[14].

Such situations called for equivalence tests. Although these were originally designed for pharmacokinetic studies[15], they have since been expanded to many areas[9,16,17]. A typical null hypothesis might assume that the means are equal, so that by disproving this hypothesis, one could conclude they were different. For an equivalence test, one assumes that the two samples are not equivalent, and by rejecting this hypothesis can conclude that they are. Here, we seek to use equivalence testing at the gene level.

Functional Analysis

Functional analysis of gene expression and other genomic data to assess enrichment, particularly biological processes or pathways, has become one of the most important methods of understanding gene expression profiles. For example, a published protocol for the DAVID Bioinformatics Resources has over 15,000 citations[3]. A paper discussing another popular approach, called Gene Set Enrichment Analysis (GSEA), has been cited over 13,000 times[1]. Perhaps the simplest way of doing such an analysis is to perform overrepresentation analysis (ORA) and test if a set of genes (perhaps those that were statistically significantly differentially expressed) overlaps a list of genes in a biological pathway more than what would be expected by chance[3,18,19]. Several important annotations of biological processes or pathways have been developed to facilitate such analyses. One of the best known is the Gene Ontology[20] (GO), although The Kyoto Encyclopedia of Genes and Genomes (KEGG)[21] and Reactome[22] have additional annotations for the relationships between genes in a pathway. The statistical significance of overrepresentation of a significant set of genes in the genes of a pathway can be tested simply using the hypergeometric test.

Other methods have been developed to improve the power and reliability of these tests, including Gene Set Enrichment Analysis (GSEA)[1]. GSEA identifies sets of genes that group together near the top or bottom of a ranked list of genes more than one would expect by chance. There is no requirement that individual genes be statistically significant by whatever metric is used. Currently, there is no approach specifically designed to identify pathways enriched for equivalent or inverse change. One possible approach is perform enrichment analysis separately for genes that are up and down regulated in each treatment and then find the intersection of pathways that move in the same or opposite directions[7,8]. nd GSEA can test for pathways that are significantly up or down regulated. However, this approach provides no indication of how likely this was to occur by chance, and perhaps more importantly, it does not indicate the degree to which pathways are changed in similar or opposing ways. We are not aware

of any methods that can specifically address these questions. Furthermore, a substantial limitation of similar approaches is the underlying assumption that biological pathways depend on the co-expression of genes in them. Some pathways likely function in this manner, and one may be able to detect sub-pathways with equivalent or inverse changes, but the results will likely be biased to simple pathways.

Methods

Approach

There are two characteristics that we would like to assess: equivalent and inverse change between treatments and controls. There are also two levels at which we want to assess these changes: the gene-level and the pathway or process-level. Here, we propose a set of statistics for assessing the degree of equivalent change and demonstrate methods of testing hypotheses related to these statistics, corresponding to these levels.

Gene-level Equivalent and Inverse Change

For gene-level changes we propose testing the equivalence of $\log_2$ fold change of gene expression. For the sake of convenience, we will assume the linear model used by Smyth, et al. (2004)[23]. This approach assumes that the data are from microarrays, given either as log-ratios of two-color arrays, or log intensities. In practice, our approach will work with any model that is able to provide $\log_2$ fold changes for gene expression and standard errors. Therefore, we make the simplifying assumption that a given $\log_2$ fold change of expression between a treatment and control follows an approximately normal distribution[24]. Although the assumption is unlikely to hold in every scenario, it can be checked where particularly important. Let β_i be the $\log_2$ fold change in gene expression between two group for gene *i.* Then, we assume

$$\hat{\beta}_i | \beta_i, \sigma_i^2 \sim N(\beta_i, \sigma_i^2)$$

and

$$s_i^2 | \sigma_i^2 \sim \frac{\sigma_i^2}{d_i} \chi_{d_i}^2$$

for s_i an estimate of σ^2 with d_i residual degrees of freedom for gene *i* in the model.

Therefore,

$$t_i = \frac{\hat{\beta}_i}{s_i/\sqrt{n}}$$

is approximately t-distributed with d_i degrees of freedom.

Next, we will assume that we have two independent experiments, which examine the same set of genes. Then, we have $\hat{\beta}_{ij}$, s_{ij}, and d_{ij} where *j* indicates the experiment. Recall that $\hat{\beta}_{ij}$ is an estimator with mean β_{ij}. Therefore,

$$s_{j_1-j_2} = s_{ij_1}/\sqrt{n_{j_1}} + s_{ij_2}/\sqrt{n_{j_2}}$$

and,

$$t_i^* = \frac{\hat{\beta}_{ij_1} - \hat{\beta}_{ij_2}}{s_{j_1-j_2}}$$

is approximately t-distributed with d_i^* degrees of freedom. When the population variances for experiments j_1 and j_2 are assumed equal, then $d_i^* = d_{ij_1} + d_{ij_2}$. If this is not the case, then we rely on Satterthwaite's correction (1946)[25]. Then

$$d_i^* = \frac{\left(\sum_{j=1}^{2} \frac{s_{ij}^2}{d_{ij} + 1}\right)^2}{\sum_{j=1}^{2} \frac{\left(\frac{s_{ij}^2}{d_{ij} + 1}\right)^2}{d_{ij}}}$$

Next, we will let Δ represent some fold change that is considered to be unimportant. Previously, we had $H_0: \beta_{ij_1} - \beta_{ij_2} = 0$. Now, we will follow Schuirmann (1987)[26] and instead consider two null hypotheses:

$$H_{01}: \beta_{ij_1} - \beta_{ij_2} \geq \Delta$$

for

$$t_i^{*1} = \frac{\hat{\beta}_{ij_1} - \hat{\beta}_{ij_2} - \Delta}{s_{j_1 - j_2}}$$

and,

$$H_{02}: \beta_{ij_1} - \beta_{ij_2} \leq -\Delta$$

for

$$t_i^{*2} = \frac{\hat{\beta}_{ij_1} - \hat{\beta}_{ij_2} + \Delta}{s_{j_1 - j_2}}$$

Rejecting H_{01} reflects confidence that the difference in effect sizes is smaller than Δ. Rejecting H_{02} reflects confidence that the difference in effect sizes is larger than $-\Delta$. Therefore, rejecting both results in a decision of statistical equivalence within the range $[-\Delta, \Delta]$. The p-value for the equivalence test should be taken as the larger of the two individual tests.

This provides us with an approach to declaring a gene *i* to have been equivalently changed across two conditions, assuming all distributional assumptions have been met. Particular care in this regard will need to be taken if the focus of a study is on a single gene. One issue with this approach as described is that for many genes there will be relatively little change in expression, but we do not want small perturbations to be considered equivalent changes. Therefore, we recommend setting a threshold for fold change that both genes must pass before being tested. This will also help to retain power across multiple tests.

This simple approach depends on nothing more than $\log_2$ fold changes and standard errors. Therefore, it should be equally applicable to microarray and RNA-seq data. We note that in most cases for which this approach will be useful, only a rough estimation of equivalently changed expression will be necessary. Therefore, the reliance on the normality of $\hat{\beta}_i$ is unlikely to be an issue.

To test for inverse change, i.e. a change that is equivalent but opposite to another, one can simply reverse the sign of $\hat{\beta}_{ij_2}$ for all *i.* Anything that is determined to be equivalent under this condition can instead be said to be inversely changed.

Equivalent Change Index

We have outlined one approach to thinking of equivalent change in gene expression values across experiments. However, it would also be useful to have a metric for the degree of equivalent change. Therefore, we introduce the idea of an Equivalent Change Index (ECI).

Let $\hat{\beta}_{ij_1}$ and $\hat{\beta}_{ij_2}$ be the effect sizes (ES) of two experiments for gene *i*. The ES could be a $\log_2$ fold change, a standardized mean difference, or a simple mean difference (although one should be consistent about their choice). Then we define the equivalent change index (ECI) for gene *i* to be:

$$\lambda_i = \frac{\operatorname{sgn}(\hat{\beta}_{ij_1} \times -\hat{\beta}_{ij_2})(\max(|\hat{\beta}_{ij_1}|, |\hat{\beta}_{ij_2}|) - (|\hat{\beta}_{ij_1}| + |\hat{\beta}_{ij_2}|))}{\max(|\hat{\beta}_{ij_1}|, |\hat{\beta}_{ij_2}|)}$$

This is simply the ratio of the smaller ES to the larger ES in terms of absolute value of both effects and with a sign reflecting whether the effects were in the same direction (positive) or opposite directions (negative). Thus, $\lambda_i \in [-1,1]$. A λ_i of 1 means that the ES was exactly the same for gene *i* in both experiments. Likewise, a λ_i of -1 means the ES was exactly opposite in both experiments. Therefore, λ_i indicates either the degree of equivalence or inverseness for a gene in one experiment compared to a separate experiment, depending on its sign.

Pathway-level Equivalent and Inverse Change

Despite the utility in being able to determine when pathways are enriched for equivalent or inversely changed genes, we are aware of no approaches specifically designed to identify them. The ECI gives us a way to deal with this situation. A high ECI indicates equivalent change, not the directionality of the change. Therefore, if some genes in a pathway are up-regulated and others down-regulated by one treatment, but the reverse happens in a second treatment, the ECI will be high for all genes in the pathway.

As an example, we can consider an experiment to determine the genomic influence of Drug B. In particular, we want to know biological functions for which Drug B has similar effects as Drug A. We measure gene expression for patients on Drug B and a control. We already have similar data for Drug A. Therefore, we calculate the ECI for Drug B and A on each gene. Next, we rank all genes by their ECI. We can now consider a hypothetical functional pathway, with a set of genes P. The full list of genes in the experiment is set G. The function $g(x)$ yields the index of a gene in G with rank x. $S = \{i|G_i \in P\}$. $R = \{i|G_i \notin P\}$. Therefore, S is the indices of the genes in the pathway, and R is the indices of the genes not in the pathway. Now, we will consider a gene of rank *x*. Suppose:

$$I_h = \begin{cases} 1 \; if \; g(h) \in S \\ 0 \; if \; g(h) \in R \end{cases}$$

$$F_R(x) = \frac{1}{n_R} \sum_{h=1}^{x} 1 - I_h$$

This gives us the proportion of genes that are not in the pathway and have a rank of x or less. We also have:

$$\hat{\lambda}_{g(h)} = \lambda_{g(h)} \left(1 - \max(p_{1,g(h)}, p_{2,g(h)})\right)$$

which yields a weighted ECI, based on the maximum p-value of the ES from each dataset for a given gene. This weight is useful to separate genes with a high or low ECI based on our confidence in their differential expression results.

$$F_S(x) = \frac{\sum_{h=1}^{x} I_h \ \left|\hat{\lambda}_{g(h)}\right|^{\omega}}{\sum_{h=1}^{n_R+n_S} I_h \ \left|\hat{\lambda}_{g(h)}\right|^{\omega}}$$

When $\omega = 0$, this gives the proportion of genes that are in the pathway and have a rank of x or less. When $\omega \neq 0$, we will get a weighted ratio, depending on $\hat{\lambda}_{g(h)}$, which we will discuss more in a minute.

Now, we can determine a quantity D:

$$D = \sup_{x} |F_S(x) - F_R(x)|$$

When $\omega = 0$, this D is the Kolmogorov-Smirnov statistic. Therefore, we could use it for a hypothesis test of whether the distribution of ranks is different for a particular pathway vs. all other pathways. Unfortunately, this is problematic. The K-S test depends on an assumption of independence, which gene expression data cannot claim. The K-S test has been shown to be sensitive to violations of this assumption, so it is important to take into account. Therefore, we will set $\omega = 1$, which will provide a weighted Kolmogorov-Smirnov statistic. Note that this is exactly the approach taken by GSEA. The difference is, we have substituted the ECI for the local statistic used by GSEA (which is based on effect size)[1]. $F_S(x)$ thus is adjusted for correlation between genes that is not associated with the treatments (this assumes that genes in a pathway would tend be correlated), because it is higher when the genes tend to be more equivalently expressed as the result of two treatments and is not a simple proportion. D nevertheless is still dependent on the size of the gene set. Therefore, we will scale D, getting $\widehat{D}$, and perform permutation testing for enrichment in the same manner as GSEA, by using the fgsea package for R. We call this approach equivalent change enrichment analysis or ECEA.

One convenient aspect of the statistic $\widehat{D}$, is that it represents a directional effect size for the entire pathway. Therefore, it can be used to judge the overall equivalent or inverse change of genes in a particular pathway, which is a particularly useful result for functional genomics. Unlike in standard GSEA, ECEA makes no assumption about the directionality of the change for gene effects. That is, genes that are up-regulated across treatments receive a high ECI, as do genes that are down-regulated across treatments. Therefore, there is no implicit assumption of co-expression for a pathway, meaning that ECEA can be used to investigate a wider array of pathways than typical GSEA, although for an entirely different use case.

Assessment

In order to assess our methods in both controlled and realistic conditions, we will examine the performance of our approach using both simulated and biological data.

Simulation

One hundred gene expression data sets, with correlation structure, were simulated as described above. Each dataset was constructed from different runs of the algorithm, using different parameters for the proportion of equivalent or inversely changed genes and then combined, each subset thus representing a pathway, with its own correlation structure. Thus, we are making the simplifying assumption that there is no correlation between pathways in the simulation and there are no overlapping genes. For each dataset, we created seven pathways with no treatment effect (to provide a background), one pathway

with equivalent change, one with inverse change, and finally a pathway without equivalent or inverse change but that was still enriched for differentially expressed genes. Varying the probability of genes being differentially expressed we ran 100 simulations at each probability level, we also varied the symmetry of changes in a pathway, and the probability of equivalent or inverse change. By symmetry, we mean proportion that were up vs down regulated. Thus, a symmetry of .5 means there was an equal probability of up vs. down regulation for differentially expressed genes. For each resulting dataset, we then calculated the number of times each equivalently or inversely changed gene was detected by our method and calculated the number of true positives (TP), false positive (FP), true negatives (TN), and false negatives (FN), which were used to calculate other statistics, such as the accuracy. We applied ECEA, GSEA, and ORA to these data and determined sensitivity and false recovery rate for the recovery of pathways with varying levels of equivalent or inversely changed genes.

Biological Data

For the biological data, we do not have a ground truth. Nevertheless, our first biological dataset examines the effect on adipose-specific Glut4 overexpression or knockout on changes in adipose tissue gene expression. These data were specifically created to have opposing effects. Finally, in our last dataset, we will examine the effects of two antidepressants, imipramine and ketamine, on the gene expression of a validated mouse model of depression. Here, our focus will be on determining whether our approach can detect previously observed commonalities in the effect of these two drugs, and hopefully shed more light on the pathways commonly affected. For each dataset, we will be examining the ability of ECEA, GSEA, and ORA to identify disease relevant pathways, as well as identifying individual genes that display equivalent or inverse gene expression patterns.

Data

For this work we will rely on three datasets: one simulated gene expression dataset, and two biological datasets.

- The simulated gene expression data was created using an approach similar to the one created by Dembele (2013)[27]. This approach simulates correlation structure between genes, like might occur in a biological pathway in real data and also allows us to simulate different treatments that affects those modules to varying degrees – thus allowing us to test both at the gene and pathway level. We modified the approach to create datasets in which genes perturbed by one treatment have a chance of being similarly perturbed (or inversely) by a second treatment.
- The first biological dataset was created by Kraus, et al.[28], to study the effect of the GLUT4 gene in adipose tissue on insulin sensitivity. These samples are available in the National Center for Biotechnology Information's (NCBI's) Gene Expression Ominbus (GEO)[29,30] under accession GSE35378. This study involved 12 mice, 3 were adipose-Glut4-/-, 3 were aP2-Cre transgenic mice (controls for the Glut4-/-), 3 were adipose-Glut4-Tg mice with Glut4 transgenically overexpressed, and 3 were FVB mice (controls for the adipose-Glut4-Tg mice). Therefore, we hypothesized we would observe more inverse than equivalent changes in this dataset when comparing changes in Glut4 knockout (KO) vs. controls to Glut4 overexpressed (OE) vs controls. Gene expression was assayed using the Affymetrix Murine Genome U74A Version 2 Array. These were background subtracted and normalized using the rma function of the oligo package[31] for the R statistical environment[32]. Differential expression was assessed using the limma package[33] for R.
- The second biological dataset was created by Bagot, et al.[34] to investigate the effect of two antidepressants on the transcriptome in a mouse model of depression. In this case, gene expression as assayed using RNA sequencing on the Illumina HiSeq 2500. Two drugs were

examined, ketamine and imipramine, and various brain regions were examined. We limited our analysis to mice that were susceptible to depression and only used samples from the prefrontal cortex (PFC), in order to control confounding and because PFC had the greatest number of these samples available. We hypothesized we would observe more equivalent changes in this dataset, as we expected the two drugs to influence similar biological pathways. Differential expression was assessed using the DESeq2[35] package for R.

Results

Simulations

The results of the simulations are shown for equivalent change in Fig. 1, and for inverse change in Fig. 2, using a probability of equivalent change or inverse change of .5 (i.e., each gene in a pathway chosen to have equivalent differential expression would have a probability of 0.5 of that equivalent change). We did 100 simulations for each set of parameters. Each simulation involved 1 equivalently changed pathway, 1 inversely changed pathway, 1 pathway with differentially expressed genes but no relationship between experiments, and 7 pathways not affected by these simulated treatments. In each case, there were 5 samples for each treatment and 5 controls (N=20). The results show the proportion of times the equivalent or inversely changed pathway was detected. The inversely changed pathway was more difficult to detect by FGSEA, because the process of enforcing the inverse change tended to increase the level of symmetry of up vs. down-regulation past the level set in the parameter. For most levels of probability of differential expression and across levels of symmetry, ECEA outperformed FGSEA or ORA. For equivalently changed pathways, FGSEA outperformed ECEA only for low levels of probability of differential expression (PDE) and when the symmetry was extreme. In Fig. 3, the false positive rate (FPR) for the pathway with differential expression but without enforced equivalent or inverse change is shown. For all levels of probability of differential expression ORA had the lowest FPR, but it is also very low for ECEA. When the symmetry is .1 or .9 the FDR is nearly linear for FGSEA, meaning that the greater the probability of differential expression, the greater the likelihood of identify a pathway as having equivalent change by chance, using this approach. Fig. 4 shows the precision and recall for gene-level tests based on an equivalence or inverseness test. In the simulated data, there was a consistently high level of precision (or positive predictive value), although sensitivity for individual genes was much lower. In this case there is no comparison method, because we are not aware of an existing method to compare to.

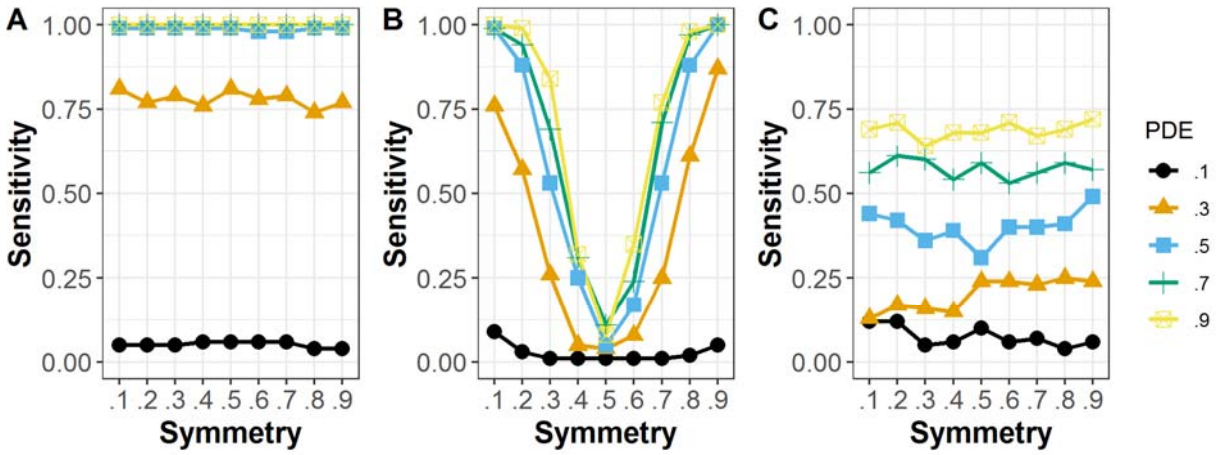

Figure 1: The proportion of equivalently changed pathways detected by each method, when the probability of equivalent change was .5. The x-axis displays the symmetry, which shows the probability of genes being up-regulated in the pathway as

opposed to down-regulated (when they were differentially expressed). With a symmetry of .5, approximately half of the differentially expressed genes would be up-regulated and the rest would be down-regulated. The different lines show the sensitivity for different levels of probability of a gene being differentially expressed. The results are shown for (A) ECEA, (B) FGSEA, and (C) ORA. For nearly all levels of probability of differential expression, ECEA was more sensitive than ORA. The FGSEA approach outperformed ECEA only for low levels of PDE and when the symmetry as extreme.

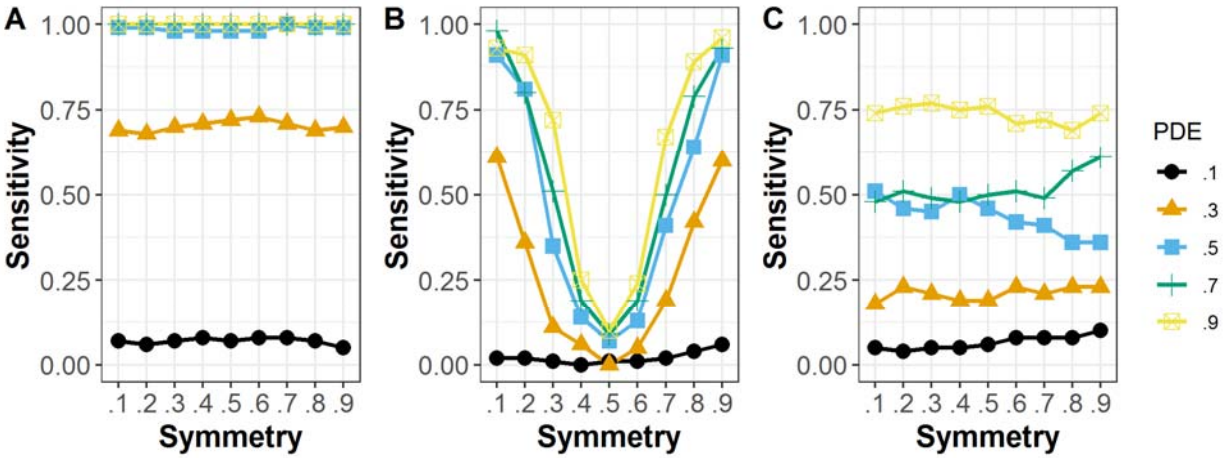

Figure 2: The proportion of inversely changed pathways detected by each method, when the probability of inverse change was .5. The x-axis displays the symmetry, which shows the probability of genes being up-regulated in the pathway as opposed to down-regulated (when they were differentially expressed). With a symmetry of .5, approximately half of the differentially expressed genes would be up-regulated and the rest would be down-regulated. The different lines show the sensitivity for different levels of probability of a gene being differentially expressed. The results are shown for (A) ECEA, (B) FGSEA, and (C) ORA. For nearly all levels of probability of differential expression, ECEA was the most sensitive.

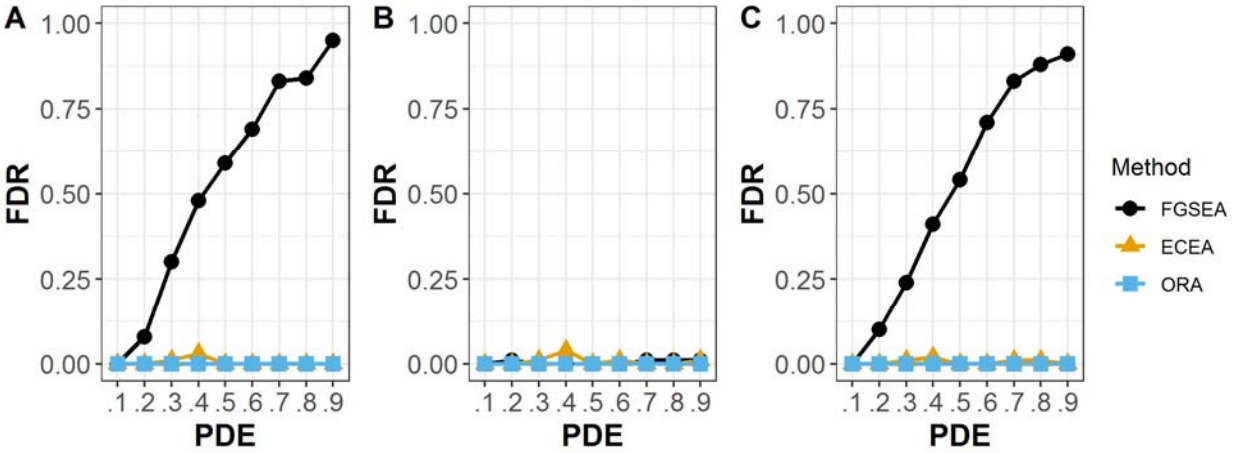

Figure 3: The proportion of times a pathway with differentially expressed genes was erroneously identified as being equivalently or inversely enriched by method. The sub-figures show the results at (A) Symmetry = .1, (B) Symmetry = .5, and (C) Symmetry = .9.

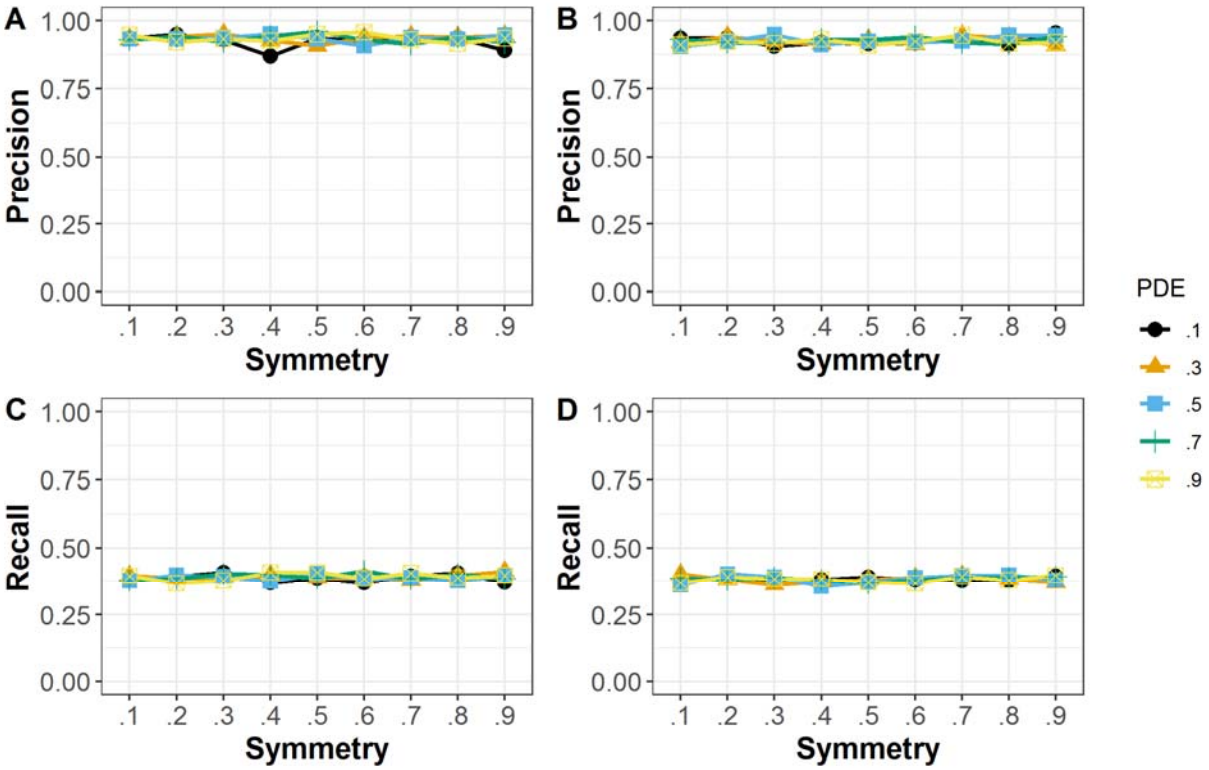

Figure 4: Mean precision and recall for individual gene-level equivalence and inverseness tests. The sub-figures refer to (A) precision for recognizing equivalently changed genes, (B) precision for recognizing inversely changed genes, (C) recall for recognizing equivalently changed genes, and (D) recall for recognizing inversely changed genes.

GLUT4 Data

ECEA was run on the GLUT4 data to determine processes and pathways enriched for genes that are equivalently or inversely changed when GLUT4 is knocked out or overexpressed in mice. The $\log_2$ fold change for each treatment vs. its respective control was calculated using the limma package for R[33]. The fold change was used to calculate the ECI, which was then used to perform the ECEA. First we performed this analysis on the Gene Ontology[20], with a false discovery rate cut-off of .2, which is within the recommended threshold for GSEA[1]. We found enrichment in equivalent or inverse change for 14 processes using this approach, which are listed in Table 1. Of those 14 pathways, 10 are enriched for inverse change. Although we would expect that more genes in this dataset will be inversely changed across the treatments, it does not necessarily follow this will be true for the pathways or process enrichment. That will depend on the representation of pathways in the dataset, the amount of overlap between pathways, and similar factors. For each enriched process, we have included the top 5 genes in the results, which are the genes with the greatest equivalent or inverse change for the respective process.

Table 1

Process	P-adj	NES	Size	Top 5 Genes
GO:0001704 formation of primary germ layer	0.092335	-1.85059	70	Mesp2,Pax2,Tlx2,Pofut2,Bmp4
GO:0001707 mesoderm formation	0.092335	-1.83538	55	Mesp2,Pax2,Tlx2,Pofut2,Bmp4
GO:0006412 translation	0.118162	1.665327	291	Rpl13a,Rps18,Rps27a,Rpl29,Eif4g1
GO:0009952 anterior/posterior pattern specification	0.118162	-1.64202	164	Hoxa5,Hoxb6,Mesp2,Bmp4,Gdf11

GO:0030029 actin filament-based process	0.184832	1.419986	280	Crk,Fbxo8,Rock2,Twf1,Cnn1
GO:0030036 actin cytoskeleton organization	0.184832	1.46935	259	Crk,Fbxo8,Rock2,Twf1,Cnn1
GO:0035115 embryonic forelimb morphogenesis	0.092335	-1.98718	22	Hoxd9,Lrp6,Wnt3,Rspo2,Msx2
GO:0035136 forelimb morphogenesis	0.156032	-1.94438	29	Gdf5,Hoxd9,Lrp6,Wnt3,Foxl1
GO:0042274 ribosomal small subunit biogenesis	0.109063	2.309349	17	Rps25,Rps16,Rps7,Rps28,Rps8
GO:0048332 mesoderm morphogenesis	0.118162	-1.83317	58	Mesp2,Pax2,Tlx2,Pofut2,Bmp4
GO:0048562 embryonic organ morphogenesis	0.092335	-1.81544	169	Rbp4,Gli1,Hoxa5,Hoxb6,Cited2
GO:0048704 embryonic skeletal system morphogenesis	0.092335	-1.80482	72	Hoxa5,Hoxb6,Mycn,Bmp4,Twist2
GO:0048705 skeletal system morphogenesis	0.092335	-1.70538	150	Thbs1,Hoxa5,Hoxb6,Cited2,Fbn2
GO:0060438 trachea development	0.092335	-1.98875	12	Hoxa5,Bmp4,Foxf1,Lrp6,Rspo2

The Gene Ontology is not an ideal dataset for a typical GSEA, because it focuses on finding enrichment in coordinate changes in gene expression. Therefore, typically curated lists of pathways are used with GSEA. Although ECEA uses the same basic methodology as GSEA, ECEA can be applied to any gene set, because it is looking for coordinate equivalently or inversely changed genes across experiments. Nevertheless, we provide a GSEA analysis using fgsea here in order to provide a comparison.

Using GSEA, for the GLUT4 KO, there were 326 significantly enriched processes. For the overexpressed data, there were 63. One way to search for equivalently or inversely changed pathways would be to find those enriched in upregulated genes using GSEA in one dataset and enriched for downregulated genes in the other (and vice versa). Between these two results, there were 38 shared pathways. Of these, 10 had an inverse relationship and 28 and an equivalently changed relationship. Naturally, using this approach there is no way to assess the statistical significance of this relationship. The inverse processes are shown in Table 2.

Table 2

Process
GO:0006635 fatty acid beta-oxidation
GO:0009063 cellular amino acid catabolic process
GO:0009081 branched-chain amino acid metabolic process
GO:0016054 organic acid catabolic process
GO:0044282 small molecule catabolic process
GO:0044712 single-organism catabolic process
GO:0044743 intracellular protein transmembrane import
GO:0046395 carboxylic acid catabolic process
GO:0065002 intracellular protein transmembrane transport
GO:0071806 protein transmembrane transport

We followed a similar approach to analyze enrichment in KEGG pathways. There are far fewer pathways in this database than there are biological processes in GO. Using ECEA, we only found five pathways with significant enrichment and only two of these were inversely enriched (Table 3).

Table 3

Pathways	P-adj	NES	Size	Top 5 Genes
mmu04141 Protein processing in endoplasmic reticulum	0.171093	1.464133	133	Hspa1b,Hspa1a,Rad23a,Skp1a,Hsp90b1
mmu03010 Ribosome	0.025988	2.902464	71	Rpl13a,Rps18,Rps10,Rps27a,Rps25
mmu00280 Valine, leucine and isoleucine degradation	0.025988	-2.06113	35	Hibadh,Bckdha,Acaa2,Mcee,Pccb
mmu00071 Fatty acid metabolism	0.171093	-1.70619	33	Acaa2,Eci1,Echs1,Acsl4,Acsl1
mmu00010 Glycolysis / Gluconeogenesis	0.066906	1.818807	53	Pgam1,Aldh1a3,Hk2,Aldoa,Hk1

Using GSEA and looking for overlapping results, we found five pathways with inverse changes in regulation, two of which were also found by ECEA. These are shown in Table 4.

Table 4

Process
mmu00280 Valine, leucine and isoleucine degradation
mmu00071 Fatty acid metabolism
mmu03320 PPAR signaling pathway
mmu04146 Peroxisome
mmu04610 Complement and coagulation cascades

It is worth noting that ECEA looks for enrichment in equivalent or inverse changes in the same genes across treatments, while this approach will simply find overall changes in genes in the pathway that tend to be in the same direction. There is no indication as to whether the functional impact is likely to be similar, such as there is for ECEA (i.e. different parts of a complex pathway might be affected and thus not result in similar functional impacts).

We hypothesized that using the gene-level significance test for equivalent or inverse change, we would see more genes with significant inverse changes than equivalent changes. This turned out to be the case. We performed the equivalence test using $\Delta = 0.5$, to both allow some latitude in differences in fold change between experiment while ensuring that we would not simply include all genes that changed in opposing directions. We looked for genes with an adjusted p-value for equivalent change of .05 or less and with an absolute $\log_2$ fold change of greater than .5. Using these criteria there was 1 equivalently changed gene and 6 inversely changed genes. The results are shown in Table 5.

Without using our approach, one could look at significantly differentially expressed genes in both datasets and look for those with inverse effects (this would still not be a test of the occurrence of this across both datasets). However, in this case, there were no genes in common across the treatments with significant differential expression. Alternatively, one might look at the top genes in terms of fold change across both experiments and look for those with inverse effects and naturally these would identify the same genes as those in Table 5 (among others), but again there would be no determination of significance. Although the ECI is not used as part of the test, it is evident from Table 5 that all the significantly inversely or equivalently changed genes have high absolute ECI scores. The distribution of ECI scores are shown in Figure 5.

Table 5

Gene	Type of Change	ECI	Adj. p-value for Equivalent/Inverse Change	Log2 Fold Change for KO	Adj. p-value for KO	Log2 Fold Change for OE	Adj. p-value for OE
Tagln	Equiv	0.952	1.45e-02	0.590	2.12e-01	0.562	9.65e-01
Alcam	Inv	-0.776	4.46e-02	-1.046	2.67e-02	0.811	7.56e-01
Hmgcs1	Inv	-0.739	1.63e-02	-0.515	9.97e-01	0.661	4.59e-01
Nid2	Inv	-0.973	3.02e-03	-0.616	4.85e-01	0.631	9.65e-01
Pth1r	Inv	-0.640	4.83e-02	0.555	1.53e-01	-0.866	2.51e-01
Rbp4	Inv	-0.880	3.02e-03	-0.652	1.58e-01	0.574	9.65e-01
Thbs2	Inv	-0.869	3.28e-02	0.547	9.97e-01	-0.594	9.65e-01

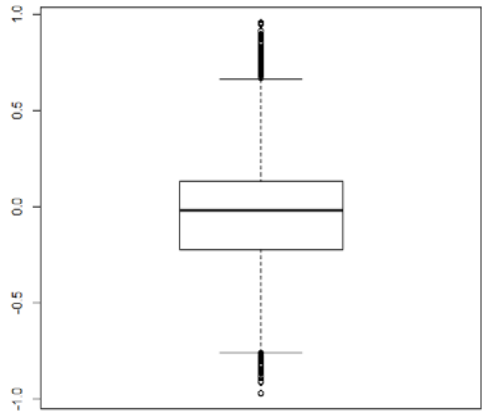

Figure 5 – the distribution of ECI scores in the GLUT4 data.

Antidepressant Data

We analyzed the antidepressant data in much the same way as the GLUT4 data, except in these data we expected we might see more equivalent than inverse changes (and we might detect similar pathways affected by two different antidepressant drugs). In this case, there were 7 processes with significant enrichment for equivalent change across treatments. Notably, 4 of these relate to circadian rhythm.

Table 6

Process	**P-adj**	**NES**	**Size**	**Top 5 Genes**

GO:0007186 G-protein coupled receptor signaling pathway	0.168385	1.190347	472	Baiap3,Mc3r,Sstr5,Crhr2,Glp1r
GO:0022410 circadian sleep/wake cycle process	0.168385	1.742638	12	Ada,Drd3,Drd2,Adora2a
GO:0035815 positive regulation of renal sodium excretion	0.168385	1.90011	10	Drd3,Drd2,Adora2a,Tacr1,Tac1
GO:0042749 regulation of circadian sleep/wake cycle	0.168385	1.787596	11	Ada,Drd3,Drd2,Adora2a
GO:0045187 regulation of circadian sleep/wake cycle, sleep	0.168385	1.787596	11	Ada,Drd3,Drd2,Adora2a
GO:0050802 circadian sleep/wake cycle, sleep	0.168385	1.742638	12	Ada,Drd3,Drd2,Adora2a
GO:1900542 regulation of purine nucleotide metabolic process	0.168385	1.219946	305	Mc3r,Crhr2,Arhgap9,Rasal3,Calcrl

When we performed GSEA for the two different treatments separately, we found 258 pathways that were significantly enriched after treatment with either drug and all 258 pathways had changes in the same direction.

For the KEGG pathways, we found 3 pathways using ECEA (Table 7). Using the GSEA overlap approach, we also found 3 pathways (Table 8), 2 of which overlaps the ECEA results.

Table 7

Pathway	**P-adj**	**NES**	**Size**	**Top 5 Genes**
mmu03010 Ribosome	0.020098	1.504855	95	Rpl3l,Rpl7a,Rpsa,Rplp1,Rpl34
mmu04080 Neuroactive ligand-receptor interaction	0.047046	1.253433	204	Mc3r,Sstr5,Crhr2,Glp1r,Chrna10
mmu00340 Histidine metabolism	0.047046	1.631414	21	Aldh3b1,Aldh7a1,Hdc,Aldh3a1,Ddc

Table 8

Pathway
mmu03010 Ribosome
mmu04370 VEGF signaling pathway
mmu04080 Neuroactive ligand-receptor interaction

For the gene-level tests there were no significant results for the specified delta and alpha levels.

Discussion

In this work we have presented a new approach to functional genomic analysis that takes into account the information available when experiments are run that could be expected to have similar or opposing effects. Analysis can be done either at the level of biological pathways/processes, or individual genes. This allows researchers an opportunity to focus particularly on changes that are extremely similar or inverted across treatments, as well as allowing for a unique form of meta-analysis that can incorporate existing, possibly publicly available data in order to gain fresh insights.

As we demonstrate, at the pathway level, a similar type of analysis can be done by intersecting the results of two separate enrichment analyses, and this is undoubtedly a useful technique. However, such approaches cannot determine if a pathway is changed in the same way (i.e. the same parts of the pathway) or to the same extent. For larger pathways, the differences may be critical. Additionally,

intersection based approaches are likely to return a greater number of results, and many of these are likely to be false positives, as shown by our simulation data. The approach outlined here can calculate the statistical significance of enrichment in genes that are dysregulated in similar or opposing ways across experiments. This should be particularly useful when applied to large pathways, because enrichment will only be found when the same parts of the pathway are affected similarly (rather than a similar trend in expression on average for the pathway overall).

A limitation in approaches that depend on co-expression of genes in a pathway for enrichment, is that they make the necessary assumption that genes are up or down-regulated together. This is the intended behavior of methods like GSEA, and is likely a useful assumption generally. But, some pathways depend on down-regulation of certain genes and up-regulation of others. For example, the apoptosis pathway in KEGG requires the down-regulation of a number of genes and the up-regulation of others during ordinary function. Our simulated data demonstrate how GSEA is quite sensitive to pathways in which genes are not co-expressed, but our approach is invariant to the direction of change in gene expression, because we are determining enrichment in similar or inverse changes across experiments. Therefore, a change can be equivalent for multiple genes, even if they are up or down-regulated in the same pathway.

In the GLUT4 dataset, which we thought of as a biological dataset with inverse changes of interest, it might appear that the pathways enriched for mostly inverse changes bear no obvious connection to diabetes, or insulin sensitivity (which is what the dataset was created to investigate and has already been confirmed by previous findings). It is important to keep in mind that these are pathways enriched for genes that are particularly equivalently or inversely regulated across treatments, and that the study was examining changes in white adipose tissue. Some of the biological processes found are related to mesoderm and skeletal system morphogenesis, thus it is interesting to note that white adipose tissue has its origins partly in the mesoderm[36], and that bone morphogenetic proteins (BMPs) can play a role in both adipogenesis and metabolism[37]. Indeed, one of the top genes driving inverse change in these pathways is Bmp4, which in humans is a regulator of adipogenesis[38], and adipocyte regulation had previously been tied to insulin sensitivity and diabetes[39]. Thus, it seems that our approach can at least identify inversely changed pathways across treatments that are relevant to the target disease, and importantly, assign a statistical significance to the results.

This is not to say that important results cannot be found using a simple overlap method with a more traditional enrichment analysis. By using GSEA to find enriched pathways that are up or down-regulated and then looking for those occurring across treatment in opposite directions we found 10 such processes. One of these is “GO:0009081 branched-chain amino acid metabolic process”, and branched chain amino acids have increasingly been thought to play a role in diabetes[40]. Naturally, we cannot know the ground truth for biological data, but we can say that both of these results are plausible, and we can say that our method is more likely to home in on results that are changed to a similar extent for the same genes. Doubtless, this will be helpful for some research efforts.

When we analyzed enrichment for equivalent or inverse changes using KEGG pathways, we again found branched chain amino acids to be enriched (valine, leucine and isoleucine degradation), using either approach. Both approaches also identified fatty acid metabolism, which is also linked to diabetes[41].

Although the ECI we use to perform ECEA does not have an associated statistical significance in its own right, we found that genes that were statistically significantly equivalently or inversely changed did tend to have high or low ECI values respectively. A number of these genes have known ties to diabetes

in humans, for example, ALCAM is a biomarker for diabetic kidney complications[42], and THBS2 has been found to associated with complications of type 2 diabetes[43]. Therefore, at the gene-level, the equivalence test also results in biologically plausible results.

In the anti-depressant data, ECEA found an enrichment for equivalent changes in circadian sleep/wake cycle. This suggests that both ketamine and imipramine have similar influence on the regulation of genes involved in circadian rhythm. Indeed, separate studies have suggested that these drugs may influence circadian rhythm[44,45]. This is suggestive of at least one mechanism of action that two different anti-depressant drugs may have in common.

This last result is exciting, because it demonstrates an important use case for our approach. Data for a new drug can be collected and commonalities in functional effects in comparison with existing drugs can be predicted, using a model organism. This has clear implications for the field of drug repositioning. One could imagine inverse enrichment could play a similarly important role, by allowing the prediction of a drug reversing the changes in genes of pathways disrupted in a disease.

A potential limitation of our approach is its strict definition of equivalent change. Likely, it would also be useful to consider non-inferiority testing, in addition to equivalence. This would test if a change was at least as large in one dataset as another, within some margin. In future work, we intend to consider just such an option.

Finally, we do not claim that this new approach should supplant existing computational functional genomics methods. Rather, we have demonstrated that it is a useful new tool that can allow researchers to garner relevant new insights into certain kinds of data. It allows for statistical rigor to be brought to research questions that are already being investigated in other ways, and potentially opens new avenues of inquiry.

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