Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox v2.0

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Over the past decade, a growing community of researchers has emerged around the use of constraint-based reconstruction and analysis (COBRA) methods to simulate, analyze and predict a variety of metabolic phenotypes using genome-scale models. The COBRA Toolbox, a MATLAB package for implementing COBRA methods, was presented earlier. Here we present a substantial update of this *in silico* toolbox. Version 2.0 of the COBRA Toolbox expands the scope of computations by including *in silico* analysis methods developed since its original release. New functions include (i) network gap filling, (ii) ^{13}C analysis, (iii) metabolic engineering, (iv) omics-guided analysis and (v) visualization. As with the first version, the COBRA Toolbox reads and writes systems biology markup language–formatted models. In version 2.0, we improved performance, usability and the level of documentation. A suite of test scripts can now be used to learn the core functionality of the toolbox and validate results. This toolbox lowers the barrier of entry to use powerful COBRA methods.

INTRODUCTION

COBRA methods have been successfully used in the field of microbial metabolic engineering[1–3] and are being extended to modeling transcriptional[4–8] and signaling[9–11] networks and in the field of public health[12]. Specifically, COBRA methods have been used to guide metabolic pathway engineering, to model pathogens[13] and host-pathogen interactions[14], and to assess the impact of disease states on human metabolism[15]. A wide variety of COBRA methods have been developed over the years[16,17]. COBRA methods have been used in hundreds of research articles over the past decade, which characterize genome-scale properties of metabolic networks and their phenotypic states[18–20].

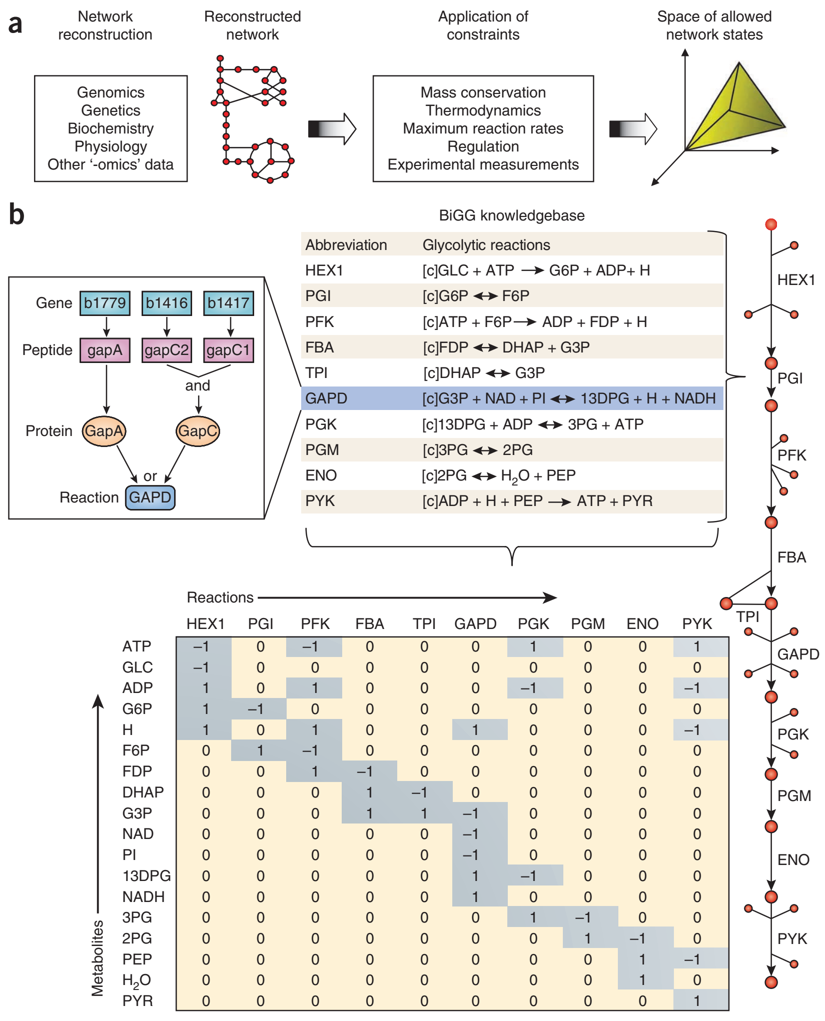
The COBRA approach focuses on using physicochemical, data-driven and biological constraints to enumerate the set of feasible phenotypic states of a reconstructed biological network in a given condition (**Fig. 1a**). These constraints include compartmentalization, mass conservation, molecular crowding[21] and thermodynamic directionality[22–24]. More recently, transcriptome data have been used to reduce the size of the set of computed feasible states[14,25,26]. Although COBRA methods may not provide a unique solution, they provide a reduced set of solutions that may be used to guide biological hypothesis development[27]. The COBRA Toolbox provides researchers with a high-level interface to a variety of COBRA methods. Detailed descriptions of COBRA methods can be found in a variety of reviews[3,16,28,29].

The biological network models that are analyzed with COBRA methods are constructed in a bottom-up fashion from bibliomic and experimental data, and thus represent biochemically, genetically and genomically (BiGG) consistent knowledgebases[30,31]. BiGG knowledgebases are manually curated 2D genome annotations[32] that relate biological functions, such as metabolic reactions, to the genome through the use of the gene-protein-reaction formalism[33] (**Fig. 1b**). Application of the BiGG formalism to metabolism has been particularly successful, and metabolic reconstructions are available for many organisms[34–42]. A detailed protocol describing the construction of high-quality BiGG knowledgebases for metabolism—and their transformation into mathematical models—has been recently published[43].

The first release of the COBRA Toolbox in 2007 provided access to a variety of methods, including flux balance analysis, gene essentiality analysis and minimization of metabolic adjustment analysis (**Table 1**). Since the release of the first version of the COBRA Toolbox, many additional COBRA-related methods have been published[44–48]. In version 2.0 of the COBRA Toolbox, we have extended the capabilities to include geometric flux-balance analysis (FBA)[44], loop law[49], creation of context-specific subnetwork models using omics data[14,25], Monte Carlo sampling[15,50–52], ^{13}C fluxomics, gap filling[45,53], metabolic engineering[46–48] and visualization of computational models of metabolism (**Table 1** and **Fig. 2**).

In addition, methods[5,24,54] and resources[55] have been developed by community members, which can serve as add-ons to the core COBRA Toolbox or provide models or other input. Specifically, Chandrasekaran and Price[54] have developed a method—probabilistic regulation of metabolism—that incorporates regulatory information from transcriptome, ChIP-chip or literature data into a metabolic network model. Fleming and Thiele[24] developed an extension to thermodynamically constrain reaction directionality, and Henry *et al.*[55] have developed a web-based resource (http://www.theseed.org/models/) that provides access to draft metabolic network reconstructions for a variety of organisms—these models may be imported into the COBRA Toolbox for further refinement and analysis.

This protocol aims to provide researchers with the ability to use the *in silico* methods included in the toolbox with only high-level knowledge of the algorithms. Because of the wide range of creative uses for COBRA methods, not all of the capabilities of the COBRA Toolbox are described in this protocol;



Abbreviation	Glycolytic reactions
HEX1	[c]GLC + ATP $\longrightarrow$ G6P + ADP+ H
PGI	[c]G6P $\leftrightarrow$ F6P
PFK	[c]ATP + F6P $\longrightarrow$ ADP + FDP + H
FBA	[c]FDP $\leftrightarrow$ DHAP + G3P
TPI	[c]DHAP $\leftrightarrow$ G3P
GAPD	[c]G3P + NAD + PI $\leftrightarrow$ 13DPG + H + NADH
PGK	[c]13DPG + ADP $\leftrightarrow$ 3PG + ATP
PGM	[c]3PG $\leftrightarrow$ 2PG
ENO	[c]2PG $\leftrightarrow$ H_2O + PEP
PYK	[c]ADP + H + PEP $\longrightarrow$ ATP + PYR

	HEX1	PGI	PFK	FBA	TPI	GAPD	PGK	PGM	ENO	PYK
ATP	–1	0	–1	0	0	0	1	0	0	1
GLC	–1	0	0	0	0	0	0	0	0	0
ADP	1	0	1	0	0	0	–1	0	0	–1
G6P	1	–1	0	0	0	0	0	0	0	0
H	1	0	1	0	0	1	0	0	0	–1
F6P	0	1	–1	0	0	0	0	0	0	0
FDP	0	0	1	–1	0	0	0	0	0	0
DHAP	0	0	0	1	–1	0	0	0	0	0
G3P	0	0	0	1	1	–1	0	0	0	0
NAD	0	0	0	0	0	–1	0	0	0	0
PI	0	0	0	0	0	–1	0	0	0	0
13DPG	0	0	0	0	0	1	–1	0	0	0
NADH	0	0	0	0	0	1	0	0	0	0
3PG	0	0	0	0	0	0	1	–1	0	0
2PG	0	0	0	0	0	0	0	1	–1	0
PEP	0	0	0	0	0	0	0	0	1	–1
H_2O	0	0	0	0	0	0	0	0	1	0
PYR	0	0	0	0	0	0	0	0	0	1

Figure 1 | The philosophy of COBRA. (**a**) COBRA of biological networks involves the creation of network models from a variety of biological data sources. The capabilities of the model are then assessed in the context of physical, chemical, regulatory and omics constraints (reproduced from Becker *et al.*[17] with permission). (**b**) COBRA models are often derived from BiGG knowledgebases, which are essentially 2D annotations of the genome that relate metabolic activity to genomic loci. (Left inset) In *E.coli*, the GAPD activity can be provided by two isozymes (GapA or GapC); GapC is a heteromeric protein that requires genes from two genomic loci. The contents of a BiGG knowledgebase can be converted to a map (right) to facilitate visual interpretation, or to a mathematical modeling formalism to develop and explore hypotheses, such as a stoichiometric matrix (bottom) that can be used to explore mass flow through the network (reproduced with permission from Reed et al.[33], with modifications).

additional functionalities are described in the Documentation.

The COBRA Toolbox supports models in the systems biology markup language (SBML) format[56]. Importing the models into MATLAB is dependent on libSBML[57] and the SBMLToolbox[58]. Because SBML does not yet provide complete support for a few key COBRA parameters, we provide an explicit description of the COBRA extensions to SBML below and in **Supplementary Discussion**. The COBRA Toolbox is available for download from http://www.cobratoolbox.org. Detailed documentation in HTML format is available in the 'docs' folder of the COBRA Toolbox.

Toolbox installation

There are two options for installing the COBRA Toolbox: 'à la carte' or bundled. The à la carte version only contains the COBRA Toolbox. The bundled version includes the COBRA Toolbox, libSBML, the SBMLToolbox, GNU linear programming kit (GLPK) and glpkmex. The bundled version has been tested on Mac OS X 10.6 Snow Leopard (64-bit), Ubuntu GNU/Linux Lucid (64-bit), Windows XP (32-bit) and Windows 7 (64-bit). Separate installation instructions are provided in Equipment Setup.

COBRA-compliant SBML file

Documentation on the SBML standard is available on the SBML website (http://sbml.org), and a description of a COBRA-compliant SBML file is provided in the **Supplementary Discussion**. Sample models in COBRA-compliant SBML may be downloaded from the BiGG knowledgebase (http://bigg.ucsd.edu)[31], or draft models may be downloaded from the Model SEED (http://www.theseed.org/models)[55]. The model files must include the following information for all calculations: stoichiometry of each reaction, upper and lower bounds of each reaction and objective function coefficients for each reaction.

Several functions within the COBRA Toolbox[47,48] require information that is not yet in the SBML standard or is scheduled for removal in SBML 3 and beyond. The gene-reaction associations are essential for relating the metabolic reactions to the genome, and the subsystem is useful for ontological classification. Metabolite formulas and charges are necessary to ensure that the model is physically consistent (no generation of mass or energy). Additional annotation parameters, such as KEGG or CAS IDs, should be specified in the notes field.

```
<reaction>
  …
  <notes>
    <html xmlns='http://www.w3.org/1999/xhtml'>
      <p>GENE_ASSOCIATION: ((gene1) and (gene2)) or
(gene3)</p>
      <p>SUBSYSTEM: Transport Inner Membrane</p>
      <p>KEGGID: …</p>
      …
    </html>
  </notes>
</reaction>
<metabolite>
  …
```

TABLE 1 | Features of the COBRA Toolbox 1.0 and 2.0.

	COBRA 1.0	New in COBRA 2.0
FBA	Growth-rate optimization Robustness analysis Gene deletion studies Flux variability MOMA	Loop law Geometric FBA
Fluxomics	None	C13 data fitting and flux estimation Experimental design
Gap filling	None	detectDeadEnds gapFind growthExpMatch
Input/output	Read/write SBML (level 2, version 1)	Read/write SBML (level 2, version 4)
Metabolic engineering	None	OptKnock OptGene GDLS
Reconstruction	Model curation tools	Create submodels using omics data
Sampling	Artificial centering hit and run (ACHR) sampling	Updated ACHR sampling (parallel/multipoint)
Test suite	None	Examples Verify installations
Visualization	None	Display maps Overlay data—flux distributions; flux variability

```
      <notes>
        <html xmlns='http://www.w3.org/1999/xhtml'>
          <p>FORMULA: C6H12O6</p>
          <p>CHARGE: 0</p>
          <p>CAS: …</p>
        …
      </html>
    </notes>
  </metabolite>
```

Metabolic map files

The visualization tools require text files of the coordinates for placing metabolites and reactions on a map. Map coordinate files for many metabolic pathways are available from the BiGG knowledgebase. The COBRA Toolbox relates COBRA SBML models to the map coordinate files through the reaction and metabolite IDs. A map file for glycolysis may be used with various SBML models as long as the identifiers match. The format for a map file is described in **Supplementary Discussion**.

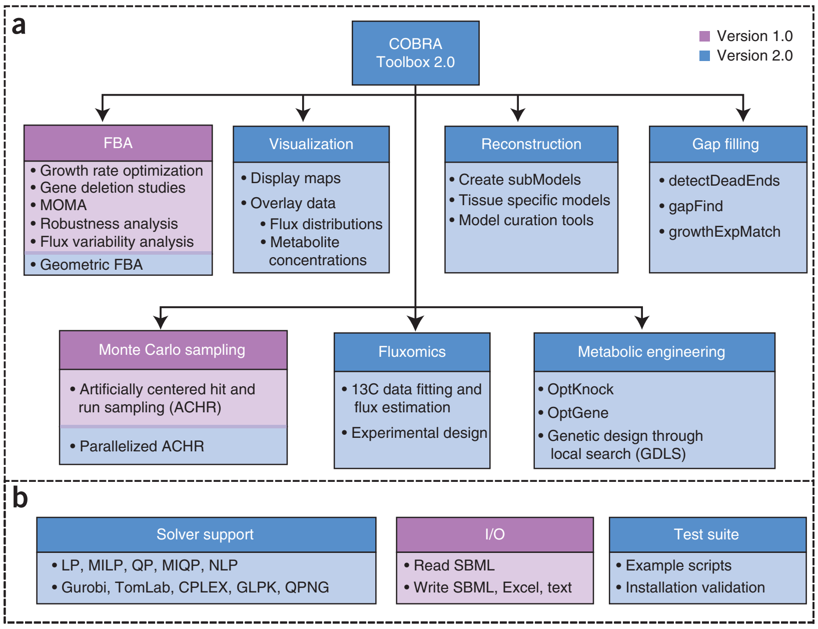


Figure 2 | Overview of the COBRA Toolbox. (**a**) Seven categories of COBRA methods contained within version 2.0 of the COBRA Toolbox. (**b**) The COBRA Toolbox contains solver interface functions for linear, quadratic, mixed integer linear and quadratic, and nonlinear programming problems. Functions to read and write models in several formats are available. A test suite is included to validate installation and provide an example for implementation of many methods.

MATERIALS

EQUIPMENT

- The COBRA Toolbox version 2.0 or above (http://www.cobratoolbox.org)
- A computer capable of running MATLAB
- Version 7.0 or above of MATLAB (MathWorks) numerical computation and visualization software (http://www.mathworks.com)
- libSBML programming library 4.0.1 or above (http://www.sbml.org)
- SBMLToolbox version 3.1.1 or above for MATLAB to allow reading and writing models in SBML format (http://www.sbml.org)
- A linear programming (LP) solver. Currently, the COBRA Toolbox supports the following:
- Gurobi (Gurobi Optimization, http://www.gurobi.com) through Gurobi Mex (http://www.convexoptimization.com/wikimization/index.php/Gurobi_mex)
- CPLEX (ILOG) through Tomlab (Tomlab Optimization, http://tomopt.com)
- GLPK (http://www.gnu.org/software/glpk) through glpkmex (http://glpkmex.sourceforge.net). Please note that GLPK does not provide accurate solutions for OptKnock or Genetic Design Local Search (GDLS) calculations as implemented in the Toolbox **! CAUTION** Other solvers (such as Mosek, http://www.mosek.com; LINDO, http://www.lindo.com; and PDCO, http://www.stanford.edu/group/SOL/software/pdco.html) may work with the COBRA Toolbox but they have not been validated. **▲ CRITICAL** For best performance, it may be necessary to adjust parameters of the installed solver.
- A quadratic programming (QP) solver (optional). Currently, the COBRA toolbox supports the following:
- CPLEX (ILOG) through Tomlab
- QPNG (part of GLPK)—please note that QPNG does not provide accurate solutions for MOMA as implemented in the Toolbox **! CAUTION** Other solvers (such as Mosek and PDCO) may work with the COBRA Toolbox but they have not been validated. **▲ CRITICAL** For best performance, it may be necessary to adjust parameters of the installed solver.
- A nonlinear programming (NLP) solver (optional). Currently, the COBRA toolbox supports:
- SNOPT through Tomlab **▲ CRITICAL** For best performance, it may be necessary to adjust parameters of the installed solver.

EQUIPMENT SETUP

À la carte installation of COBRA Toolbox

- Install MATLAB
- Install libSBML, the SBML Toolbox and selected solvers according to their specific instructions.
- Unpack the COBRA 2.0 archive

Bundled installation of COBRA Toolbox

- Install MATLAB
- Unpack the COBRA 2.0 archive. Cobra_Install_Path is the path to the top-level directory for the COBRA Toolbox
- Update Shared Library Path (Mac OS X and GNU/Linux only). For Mac OS DYLD_LIBRARY_PATH = Cobra_Install_Path/external/toolboxes/SBMLToolbox_3.1.2/toolbox/. For GNU/Linux LD_LIBRARY_PATH = Cobra_Install_Path/external/toolboxes/SBMLToolbox_3.1.2/toolbox.

PROCEDURE

Initializing the COBRA Toolbox

▲ CRITICAL *Italics* denotes a *parameter* that is supplied to a function. A bracketed *[parameter]* is optional. The symbol > > denotes the MATLAB command line; anything directly following > > is meant to be entered on the command line. All time estimates for the functions are predicated on a model using ~1,200 genes, 2,300 reactions, 1,800 metabolites and a computer with a 2.4-GHz Intel Core 2 Duo processor. When substantial preprocessing efforts are required, we provide time estimates based on personal experience.

1| Navigate to the directory where you installed the COBRA Toolbox:
> > initCobraToolbox()

2| Save the paths added if desired:
> > savepath()

Changing COBRA solvers

3| Set the solvers used by the COBRA Toolbox using the following function:
> > changeCobraSolver(*solverName*, *[solverType]*);

Variables are defined as follows: *solverName* specifies the solver package to be used; the COBRA Toolbox currently supports 'gurobi', 'tomlab_cplex', 'glpk' and 'qpng'. *solverType* (default 'LP') specifies the type of problems ('LP', 'MILP', 'QP', 'MIQP', 'NLP') to solve with the solver specified by *solverName*. When changeCobraSolver is called without any arguments, it will return the names of the current settings of the solvers.

Run COBRA Toolbox test suite ● TIMING ~10^3 s

4| The test suite contains scripts that test the functionality of scripts within the COBRA Toolbox. The scripts in the testing directory provide useful examples of many of the functions of the toolbox.
> > testAll()

testAll sequentially navigates the test suite directory (testing) and runs each test. Upon completion, it displays those tests that were completed successfully and those that failed.

! CAUTION For solver suites other than Gurobi or Tomlab, the user may encounter failures that require tuning of solver parameters.

? TROUBLESHOOTING

Read COBRA-compliant SBML models into MATLAB ● TIMING ~10^2 s

5| Load a COBRA-compliant model into MATLAB. To load a model, navigate within MATLAB to the directory containing the model and call the following function from the command window:

```
>> model = readCbModel([filename]);
```

When called with no arguments, readCbModel will prompt the user to select a file using a dialog box. readCbModel supports SBML-formatted (level 2, version 1 or 4) files. SBML files for a variety of organisms are available from the BiGG knowledgebase (http://bigg.ucsd.edu)[31]. The function returns a COBRA Toolbox model structure containing the necessary fields to describe the model for use with subsequent steps. See **Supplementary Discussion** for a description of the fields in a COBRA Toolbox model structure; hereafter, *model* denotes a COBRA Toolbox model structure.

▲ CRITICAL STEP If the model is not properly loaded into MATLAB, none of the following functions will work. Ensure that libSBML and SBML Toolbox are properly installed and are accessible by MATLAB, and that the SBML file is formatted correctly.

Saving the model

6| COBRA Toolbox model structures may be saved as text or SBML files. On Microsoft Windows, the structures may also be written to an Excel (.xls) file.

```
>> writeCbModel(model, format, [fileName], [compSymbolList], [compNameList], [SBMLLevel], [SBMLVersion]);
```

For *format*, use 'sbml' for SBML file format or 'xls' for Excel format (only available on MS Windows). For *filename*, use the name of the file. If it is not provided, a dialog box will prompt the user to specify the name and location of the output file. This feature is dependent on the SBML Toolbox to generate the XML file. The toolbox is able to output SBML level 2, version 1 or 4.

Modify COBRA Toolbox models

7| Once the model is loaded into MATLAB by readCbModel, the model can be modified to simulate different conditions, such as altering reaction bounds (A), adding (B) or removing reactions (C) or changing the model objective (D).

(A) Alter reaction bounds

(i) To alter reaction bounds, use the following function:

```
>> model = changeRxnBounds(model, rxnNameList, value, boundType);
```

Where *rxnNameList* is a cell array of reaction IDs corresponding to reaction IDs in *model.rxns*; *value* is a floating point number; *boundType* specifies the bounds that should be changed for the reactions and can take values of 'l', 'u' or 'b' for lower, upper or both, respectively. This function is useful for defining the *in silico* media composition by changing the lower bounds of exchange reactions.

(B) Add new reactions

(i) New reactions can be added to a COBRA Toolbox model using the following function:

```
>> [model] = addReaction(model, rxnName, metaboliteList, stoichCoeffList, [revFlag], [lowerBound], [upperBound], [objCoeff], [subsystem], [grRule], [geneNameList], [systNameList], [checkDuplicate]);
```

metaboliteList is a list of metabolites involved in the reaction (if a metabolite does not exist in *model.mets*, then this function will add it); *stoichCoeffList* is a list of the stoichiometric coefficients for the corresponding elements in *metaboliteList*. This function checks for reactions with the same name or stoichiometic coefficients; however, this can be disabled by setting *checkDuplicate* to false.

(C) Remove reactions

(i) To remove a reaction, call the following function:

```
>> [model] = removeRxns(model, rxnRemoveList)
```

Where *rxnRemoveList* is a cell array of reaction IDs corresponding to elements in *model.rxns*. Metabolites that are no longer involved in any reactions are removed from the *model*. The model may no longer function after reactions have been removed.

(D) Change the model objective

(i) COBRA modeling often entails performing calculations that focus on a specified objective, such as growth[59]. To change the objective function, use the following function:

```
>> model = changeObjective(model, rxnNameList, [objectiveCoeff]);
```

Where *rxnNameList* is either a string or a cell array of strings containing reaction IDs corresponding to elements in *model*.rxns that should be included in the objective function; *objectiveCoeff* specifies the weight given to the respective reaction in *rxnNameList*. If left empty, *objectiveCoeff* is assumed to be 1.

Omics-guided creation of context-specific models ● TIMING ~10^2 s plus > 1 h to format data

8| An emerging application of genome-scale reconstructions is analyzing omics data in a systems context[14,25,26]. In particular, this procedure is useful for building cell-, tissue- or condition-specific models. createTissueSpecificModel is designed to map transcriptomic or proteomic data onto a reconstruction, using two established algorithms (GIMME[25] or iMAT[26]).

The GIMME algorithm is an LP procedure that best matches high-throughput data to an original flux distribution derived from the full model; thus, the algorithm requires a predefined objective function. The iMAT algorithm is an MILP procedure that best matches high-throughput data to pathway length, thus avoiding the need for a predefined objective function. Novice users can utilize the GIMME algorithm with two inputs: the COBRA model and expression data, whereas more experienced users can tweak additional parameters.
>> [tissueModel,Rxns] = createTissueSpecificModel(*model*,*expressionData*,*[proceedExp]*,*[orphan]*,*[exRxnRemove]*,*[solver]*,*[options]*,*[funcModel]*);
Required inputs: *model* is a reconstruction with gene-protein-reaction associations; *expressionData* is a structure that contains two attributes: *.Locus* (a vector of GeneIDs matching gene IDs in *model.genes*) and *.Data* (a vector of presence/absence calls).
Optional inputs: *proceedExp* (default value of 1; to process multiple data sets, set *proceedExp* to 0); *orphan* (default value of 1) controls whether reactions with no known gene-protein-reaction association are included when performing iMAT-based network trimming (orphan reactions are always included when the GIMME method is used, regardless of the *orphan* setting); *exRxnRemove* is a list of select exchange reactions that are excluded (that is, if a specific cell or tissue is known not to have a particular metabolite transporter); *solver* is either 'GIMME' or 'iMAT' and defaults to 'GIMME'; *options* is only used with the GIMME algorithm, and it specifies the reactions that comprise the objective function (by default, the objective function is chosen from *model.c* with a 90% (0.9) threshold); *funcModel* controls whether the output *tissueModel* is fully functional (every reaction can carry a flux) or not when using the GIMME algorithm.
Output: *tissueModel* is the final cell-, tissue- or condition-specific model generated from the function; *Rxns* is a structure containing statistics regarding the reactions that were or were not expressed based on the omics data and the reactions that were added or removed from the model (see ANTICIPATED RESULTS).

Visualization ● TIMING ~10^1 s

9| Visual representation of a metabolic network can aid in understanding the model. Maps for a variety of metabolic pathways are available for many of the models hosted in the BiGG knowledgebase (http://bigg.ucsd.edu). See **Supplementary Discussion** for a description of the map file format. These maps may be used for other organisms that have similar metabolic pathways, given that the user uses the same metabolite and reaction IDs as the BiGG model that was used to create the map. To load a map, the following command is used:
>> map = readCbMap(*[filename]*)
If readCbMap is called with no arguments, a dialog box will prompt the user to select a map file. After the map has been read into MATLAB, it can be viewed as a MATLAB figure or a scalable vector graphic (svg).

10| To view a map as a MATLAB figure, the following commands are used:
>> changeCbMapOutput('matlab')
>> drawCbMap(*map*,*[options]*,*[varargin]*)
Where *options* is a map options structure created by setMapOptions. See software documentation for description of optional parameters and ANTICIPATED RESULTS for an example.

11| To save a map as an SVG file, the following commands are used:
>> changeCbMapOutput('svg');
>> drawCbMap(*map*,*[options]*,*[varargin]*)
By default, drawCbMap will create a file named 'target.svg' in the current working directory. The filename can be set by inputting additional parameters:
>> drawCbMap(*map*,'FileName',*filename*)

Simulate optimal growth using FBA ● TIMING < 10^2 s

12| Simulating optimal growth using FBA is one of the fundamental COBRA phenotypic calculations for metabolic network models. FBA is a method that calculates the flow of metabolites through a metabolic network[28]. Growth is simulated by optimizing the model for flux through the biomass function of the model; however, it is also possible to perform simulations that focus on optimizing other biological characteristics, such as ATP production. The reaction to optimize is set using the *model.c* vector (see Step 7D).
In addition to specifying an objective, it is also necessary to define the *in silico* growth medium; this is accomplished by modifying the bounds of exchange reactions. Exchange reactions for metabolites comprising the *in silico* growth medium should have a lower bound less than 0; all other exchange reactions should have a lower bound of 0. All exchange reactions should have an upper bound greater than 0 to prevent metabolite buildup. The solution returned will have units based on the units used in the model (typically mmol per gDW per h). FBA can be performed either in (A) standard or (B) geometric mode:

(A) Standard mode

(i) Standard FBA is performed as follows:

```
>> [solution] = optimizeCbModel(model, [osenseStr], [minNorm], [allowLoops])
```

Where *osenseStr* is either 'max' or 'min' to maximize or minimize the value of the objective, respectively; *minNorm* (default 0, if non-zero, attempt to find a solution that minimizes the presence of loops); *allowLoops* (default *true*; if set to *false*, use the loop law algorithm[49] to remove loops—this procedure can be time-consuming).

optimizeCbModel will return a solution structure containing the objective value 'f', the primal solution 'x', the dual solution 'y', the reduced cost 'w', a universal status flag 'stat', a solver-specific status flag 'origStat' and the time to compute the solution 'time'. The primal solution, 'x', represents the flux carried by each reaction within the model. The dual solution, 'y', represents the shadow prices for each metabolite and indicates the extent to which the addition of the corresponding metabolite will increase or decrease the objective value[28,60]. The reduced cost, 'w', indicates the extent to which each reaction affects the objective. A solver status of 1 indicates that an optimal solution was found.

(B) Geometric mode

(i) Geometric FBA[44] is an alternative to standard FBA. Geometric FBA attempts to return the minimal flux distribution central to the bounds of the solution space while still maintaining optimal growth rate. The flux distribution returned should then be reproducible regardless of the solver used.

```
>> flux = geometricFBA(model,[varargin])
```

The function returns the vector 'flux', which contains the centered optimal flux distribution.

13| To visualize an optimal flux distribution, the optimal flux distribution obtained using optimizeCbModel or geometricFBA can be overlaid onto an existing map of the model using the following function:

```
>> drawFlux(map, model, flux, [options], [varargin])
```

Where *map* is a map object created with readCbMap (see Visualization, Step 9); *model* is the COBRA model structure that was used for performing FBA or Geometric FBA; *options* is a drawCbMap options structure.

14| To classify model genes on the basis of an optimal FBA solution, parsimonious FBA (pFBA) is an FBA approach that incorporates flux parsimony as a constraint to categorize the solution space[61]. The concept of flux parsimony, in the context of a metabolic network, refers to minimizing the total material flow required to achieve an objective.

In this method, genes are classified into six categories: (i) essential genes (i.e., metabolic genes necessary for *in silico* growth in the given media); (ii) pFBA-optima genes (i.e., non-essential genes contributing to the optimal growth rate and minimum gene-associated flux); (iii) enzymatically less-efficient genes, which require more flux through enzymatic steps than through alternative pathways that meet the same predicted growth rate; (iv) metabolically less-efficient genes requiring a growth rate reduction if used; (v) pFBA no-flux genes that are unable to carry flux in the experimental conditions; and (vi) blocked genes, which are only associated with the reactions that cannot carry a flux under any condition ('blocked' reactions).

To categorize the genes and reactions within a model and return a model with flux minimization constraints, execute the following:

```
>> [GeneClasses, RxnClasses, modelIrrevFM] = pFBA(model, [varargin])
```

Where GeneClasses contains a list of all genes that are within the categories above; RxnClasses contains a list of all reactions that are within the categories above; and modelIrrevFM is a model that contains the flux minimization constraints. If a map is available for the model, the results from this function can be visualized by using the 'map' and 'mapoutname' flags in the varargin input. A test case may be found in the ANTICIPATED RESULTS section. Additional options are described in the software documentation directory.

▲ **CRITICAL STEP** The subsequent steps in this protocol rely on the functionality of optimizeCbModel. If optimizeCbModel fails to return a feasible flux distribution for the examples within this protocol, the problem may be due to the installation of the LP solver. It is not necessary that geometricFBA return a solution for the subsequent steps.

Solving COBRA problem structures (advanced user) ● **TIMING** > 10^0 s

15| The COBRA toolbox has five function calls used for solving different optimization problems. Basic users will not need to call these low-level functions directly, as higher-level functions encapsulate these calls. These functions act as a common interface for different LP, MILP, QP, MIQP and NLP solvers, thus ensuring that laboratories can share code even when using different installed solvers.

The five solver functions use a similar input argument structure: problem structure followed by optional argument/value pairs. The required fields in the problem structure vary for each function to supply the required information to solve the type of problem. For example, the mixed integer problem structures require a field that specifies variable type (continuous, integer, binary). A description of the format of COBRA problem structures can be found in **Supplementary Discussion**. The COBRA solution structure also provides a common output format regardless of the solver used.

```
>> [solution] = solveCobraLP(LPproblem, [varargin])
>> [solution] = solveCobraMILP(MILPproblem, [varargin])
>> [solution] = solveCobraQP(QPproblem, [varargin])
>> [solution] = solveCobraMIQP(MIQPproblem, [varargin])
>> [solution] = solveCobraNLP(NLPproblem, [varargin])
```

Simulating deletion studies ● TIMING ~10^2–10^4 s

16| Deletion studies can be easily simulated with *in silico* models. Gene deletion methods within the COBRA Toolbox are dependent on the proper setup of the gene-reaction matrix, as well as on the rules defining the Boolean relationship between genes and reactions. Reactions that are affected by a gene deletion have their upper and lower flux bounds set to zero, and are therefore not functional. The set of reactions on which a gene deletion has an effect is calculated using the gene reaction association and rules.

It is possible to study either (A) single essential gene deletions or (B) pairs of synthetic lethal genes. The possible results from deletion studies are unchanged maximal growth, reduced maximal growth or no growth (lethal). Deletion studies can be used to predict gene/reaction essentiality.

(A) Essential gene study

(i) Apply the following code:

```
>> [grRatio, grRateKO, grRateWT, hasEffect, delRxns, fluxSolution] = singleGeneDeletion(model, method, [geneList])
```

Where *method* can be either 'FBA' (default), 'MOMA'[62] or linear MOMA ('lMOMA'); *geneList* is a cell array of genes corresponding to model.genes (if not provided, deletion simulations are performed for all genes in the model); grRatio is the growth rate of the knockout/growth rate of wild type (WT); grRateKO is the growth rate of the knockouts; grRateWT is the WT growth rate; hasEffect is a Boolean list indicating whether deletion of the corresponding gene alters the growth rate; delRxns contains a list of the reactions, the bounds of which are set to 0 for each gene deletion; and fluxSolution is the flux solution for each deletion.

(B) Synthetic lethal study

(i) Apply the following code:

```
>> [grRatioDble, grRateKO, grRateWT] = doubleGeneDeletion(model, method, [geneList1], [geneList2])
```

Where *method* can be either 'FBA' (default), 'MOMA'[62] or linear MOMA ('lMOMA'); *geneList1* is a cell array of genes corresponding to model.genes (if not provided, the function assumes all genes in *model.genes* are to be interrogated); *geneList2* is a cell array of genes that correspond to the second set of genes in the synthetic lethal pair (if not provided, the function assumes that all genes in *model.genes* are to be interrogated); grRatioDble is the growth rate of the knockout/growth rate of WT; grRateKO is the growth rate of the knockouts; and grRateWT is the WT growth rate.

Flux variability analysis (FVA) ● TIMING ~10^2 s

17| Flux balance analysis only returns a single flux distribution that corresponds to maximal growth under given growth conditions. However, alternate optimal solutions may exist, which correspond to maximal growth. FVA calculates the full range of numerical values for each reaction flux within the network[63].

To determine the minimum and maximum flux values that the reactions within the model can carry, while obtaining a specific percentage of optimal growth rate, the following function is used:

```
>> [minFlux, maxFlux] = fluxVariability(model, optPercentage, [rxnNameList], [verbFlag], [allowLoops])
```

Where *optPercentage* (default 100) specifies the percentage of optimal that an alternate flux distribution must realize in order to be considered an acceptable alternative flux distribution.

Visualization of FVA results

18| To visualize the results from this function, a flux variability map can be generated from an existing reaction map, thereby color-coding reactions based on flux directionality.

```
>> drawFluxVariability(map, model, minFlux, maxFlux, [options])
```

Where *map* is the map structure corresponding to the model read in using readCbMap; *model* is the COBRA model structure used in the fluxVariability function; *minFlux* and *maxFlux* are vectors generated by the fluxVariability function described above; and *options* is a structure containing optional parameters such as edge and node color and size: bidirectional reversible reactions are colored green, unidirectional reversible reactions that carry flux in the forward direction are colored magenta, unidirectional reversible reactions that carry flux only in the reverse direction are colored cyan and irreversible fluxes are colored blue.

Sampling the solution space (advanced user) ● TIMING $>10^2$ s

19| FBA only returns a single optimal point and thus yields little information about the entire solution space. An alternative approach is to characterize the solution space using sampling[27]. The generalized parallel sampler samples can be assigned to any arbitrary linearly constrained space by moving a fixed number of points in parallel.

```
>> [sampleStructOut, mixedFrac] = gpSampler(sampleStruct, [nPoints], [bias], [maxTime], [maxSteps])
```

Where *sampleStruct* is the COBRA Toolbox problem structure for LP problems (see **Supplementary Discussion**); *nPoints* is the number of sampling points; *maxTime* is the maximum sampling time; *bias* is a structure that imposes marginal distributions on reactions; sampleStructOut is *sampleStruct* with the addition of the 'points' field containing the solutions; and mixedFrac gives an estimate of the extent to which the sampling solution is mixed relative to the warm-up points—a mixedFrac value of 0.5 indicates complete mixing.

Fluxomics (advanced user) ● TIMING $>10^2$ s

20| Carbon-13 (C13) tracing experiments provide the ability to measure internal flux rates in a metabolic network[64]. To use this data, additional information regarding carbon tracking must be added to the COBRA model. This is stored in the .isotopomer field as described in **Supplementary Discussion** (Section S.4.). To use the C13 solver, the functions must be generated:

```
>> [experiment] = generateIsotopomerSolver(model, inputMet, [experiment], [FVAflag])
```

Where *model* is the COBRA model with an .isotopomer field; *inputMet* is a string corresponding to the C13-labeled input; experiment is a list of metabolites that must be measured; and *FVAflag* removes reactions that cannot carry a flux.

21| Two solvers are generated, one based on the cumomer method[65] and one on the faster elementary metabolite unit method[66]. The solvers are called internally during the scoreC13Fit function below. A given flux distribution can be scored against a set of C13 data:

```
>> output = scoreC13Fit(v0,expdata,model)
```

Where *v0* is the initial guess for fitting, and *expdata* is one or more sets of experimental data described in **Supplementary Discussion** Section S.3.

22| Next, the most optimal flux distribution can be found with a nonlinear optimization:

```
>> [vout] = fitC13Data(v0,expdata,model, [majorIterationLimit])
```

This function will return the flux with the lowest experimental score found by the NLP solver. Very often it is useful to compute the confidence intervals of reactions, which are consistent with C13 data.

```
>> [vs, output, v0] = C13ConfidenceInterval(v0, expdata, model, max_score, [directions], [majorIterationLimit]) (~10^2 s)
```

Where *v0* is the initial guess; *expdata* is the experimental data that must be fit; *max_score* is the highest acceptable score; and *directions* is the list of reactions and reaction ratios that will be maximized and minimized (by default all reactions).

Gap filling ● TIMING ~10^3 s

23| Because of incomplete knowledge, a metabolic model may possess gaps. A gap is defined as missing biochemical information that can explain discrepancies between model predictions and experimental data. Gaps are typically the reactions that facilitate the conversion of an available metabolite in the model to one that is necessary to achieve an objective. Identifying gaps in metabolic models can be attempted using either (A) detectDeadEnds or (B) gapFind.

(A) Detect dead ends in a model

(i) Apply the following code:

```
>> outputMets = detectDeadEnds(model, [removeExternalMets])
```

The detectDeadEnds function searches the *model.S* matrix for metabolites that participate in only one reaction (can either be produced or consumed) and returns the corresponding indices for the metabolites in the *model.mets* field. Setting *removeExternalMets* to true removes external metabolites from the results. Not all gaps can be identified by simply inspecting the *model.S* matrix.

(B) Find all gaps in a model

(i) The GapFind algorithm[45] allows one to find all gaps in a model and all metabolites that are downstream from a model gap:

```
>> [allGaps, rootGaps, downstreamGaps] = gapFind(model, findNCgaps, verbFlag)
```

Where allGaps is a list of the metabolite indices for a metabolite at a gap; rootGaps is a list of metabolites that cannot be produced; and downstreamGaps is a list of metabolites that are produced in a reaction that requires a metabolite that cannot be produced.

This function is run in an interactive and iterative manner to guarantee that all gaps are identified. Set the lower bound of all exchange reactions within *model* to -1, the upper bound of all reactions to a relatively large positive number (for example 10^5) and the lower bound of all reversible reactions to a relatively large negative number (for example -10^5) within *model*. The appropriate bound magnitude required varies from model to model. If the bound magnitudes are too small, the algorithm will incorrectly identify many metabolites as gaps; if this occurs, increase the bound magnitudes by tenfold. Repeat this process as necessary until the algorithm does not identify all metabolites as gaps.

24| In addition to these two gap identification functions, the COBRA Toolbox includes an optimization-based algorithm (growthExpMatch) that identifies candidate reactions to fill gaps in the model[53]. growthExpMatch identifies the minimum number of reactions from a universal reaction database that are required for a metabolic model to grow on a specified substrate.
>> [solution] = growthExpMatch(*model*, *KEGGFilename*, *compartment*, *iterations*, *dictionary*, *logFile*, *threshold*)
Where *KEGGFilename* is the name of the reaction .lst file downloaded from KEGG (http://www.genome.jp/kegg)[67,68]; *compartment* is a string denoting for which compartment to generate exchange reactions; *iterations* controls the number of iterations to run the function; *dictionary* is an *n* x 2 cell array that maps metabolites to KEGG IDs; *logFile* is the name of the .mat file to save the solution to; and *threshold* is the minimum value that the biomass function can take for the model to be considered growing.

25| Display the growthExpMatch solution by printing the log file using the following function:
>> printSolutionGEM(*matrixSUX*, *solution*)
Where *matrixSUX* is generated with the generateSUXMatrix function, and where *solution* is the solution generated in Step 24.

Metabolic engineering ● TIMING 10^2–10^3 s
26| The COBRA Toolbox version 2.0 provides three methods for *in silico* metabolic engineering: (A) OptKnock[46], (B) OptGene[47] and (C) GDLS[48].
(A) OptKnock
(i) OptKnock runs the OptKnock algorithm[46] to determine reaction sets to knock out for the overproduction of a specific product when the model is optimized for internal cellular objectives.
>> [OptKnockSol, biLevelMILPproblem] = OptKnock(*model*, *selectedRxnList*, *[options]*, *[constrOpt]*, *[prevSolutions]*, *[verbFlag]*)
Where OptKnockSol contains the best knockout set, and biLevelMILPproblem is the MILP problem generated by the algorithm and subsequently solved. See ANTICIPATED RESULTS for an example setup of *options* and *constrOpt* structures.
There are several points to take note of when calling the OptKnock function. First, the function does not use the upper and lower bounds set within the model that is passed in. The model is first converted into irreversible format, splitting reactions with a lower bound <0 and upper bound >0. The resulting set of reactions has its lower bounds set to 0 and upper bounds set to *options.vMax*. Use the *constrOpt* structure to apply constraints on reactions, such as a minimal flux through the biomass function or ATP maintenance. Failure to set the proper constraints may lead to incorrect predictions generated by the function.
(B) OptGene
(i) OptGene is an evolutionary programming-based method to determine gene knockout strategies for overproduction of a specific product[47]. It can handle nonlinear objective functions such as product flux multiplied by biomass.
>> [x, population, scores, optGeneSol] = OptGene(*model*, *targetRxn*, *substrateRxn*, *generxnList*, *maxKOs*, *[population]*)
Where *targetRxn* specifies the reaction to optimize; *substrateRxn* specifies the exchange reaction for the growth; *generxnList* is a cell array of strings that specifies the genes or reactions that are allowed to be deleted; and *maxKOs* sets the maximum number of knockouts; x is the best scoring set as determined by the functions optGeneFitness or optGeneFitnessTilt; population is the binary matrix representing the knockout sets; and optGeneSol is the structure summarizing the results. If resuming a previous simulation, the binary matrix (*population*) can be specified.
(C) Genetic design local search
(i) The GDLS algorithm[48] may be used to identify what to knock out in order to increase *in silico* production of desired metabolites
>> [gdlsSolution, biLevelMILPproblem, gdlsSolutionStructs] = GDLS(*model*, *targetRxns*, *[vargin]*)
Where *targetRxns* is a specific list of genes, gene sets or reactions to delete; gdlsSolution is the knockout solution; biLevelMILPproblem is the bilevel MILP problem for the solution; and gdlsSolutionStructs contains the intermediate solutions. This approach typically runs faster than the global search performed by OptKnock; however, it is not guaranteed to identify the global optima.
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice for several steps in the protocol can be found in **Table 2**. If your problem is not addressed here, see The openCOBRA Project website (http://opencobra.sourceforge.net/) for discussion forums and support. See also Documentation.

TABLE 2 | Troubleshooting table.

Problem	Solution
Tests failed in testing suite (it is not necessary to pass all tests to use the COBRA Toolbox)	Check that all necessary requirements are met for the function to run: Is the correct solver selected/installed? Are libSBML 4.0.1+ and SBMLToolbox 3.1.1+ properly installed and accessible by MATLAB? Are all toolbox functions included in the MATLAB path? Are any of the COBRA toolbox functions shadowed by a function with the same name?
SBML file not read correctly	Check that libSBML 4.0.1+ and SBMLToolbox 3.1.1+ are installed and accessible in MATLAB. Is the file a COBRA compliant SBML file? (see **Supplementary Discussion**) If compartment abbreviations are not used as compartment IDs within the file, specify all compartment abbreviations using compSymbolList input in readCbModel
Unknown compartment error using writeCbModel	Specify all compartment abbreviations and names using compSymbolList and compNameList respectively when calling writeCbModel
drawCbMap requires considerable amount of time to produce output	Try changing output to SVG. Text rendering in MATLAB figures is time consuming
optimizeCbModel returns an infeasible or infinite solution	For an infeasible solution, check that the constraints on the model allow experimental growth. For an infinite solution, check that at least one constraint is limiting
solveCobraLP/MILP/QP/MIQP fails because csense or vartype is not a character array	Be sure that csense and/or vartype is initialized as a character array. MATLAB may cast the variable as a double instead. Run verifyCobraProblem to verify the format of the problem structure

Additional troubleshooting solutions can be found at the COBRA toolbox Google group (http://groups.google.com/group/cobra-toolbox).

● TIMING

Step 4, Run COBRA Toolbox test suite: ~10^3 s
Step 5, Read COBRA-compliant SBML models into MATLAB: ~10^2 s
Step 8, Omics-guided creation of context-specific models: ~10^2 s plus > 1 h to format data
Steps 9–11, Visualization timing: ~10^1 s
Step 12, Simulate optimal growth using FBA: <10^2 s
Step 15, Solving COBRA problem structures: >10^0 s
Step 16, Simulating deletion studies: approximately 10^2–10^4 s
Steps 17 and 18, Flux variability analysis: ~10^2 s
Step 19, Sampling the solution space: >10^2 s
Steps 20–22, Fluxomics: >10^2 s
Steps 23–25, Gap filling: ~10^3 s
Step 26, Metabolic engineering: approximately 10^2–10^3 s

ANTICIPATED RESULTS

Here we provide examples of COBRA Toolbox functionality that is commonly used for our research or by external collaborators. For the most part, the default settings for the functions will suffice. Advanced users interested in additional features of the COBRA Toolbox should explore the documentation in the COBRA Toolbox, as well as the forums and bug-trackers available at http://opencobra.sourceforge.net.

Displaying and saving metabolic maps

The format for a metabolic map coordinate file is described in **Supplementary Discussion**. Maps are available for download from the BiGG database (http://bigg.ucsd.edu/).

Load a map coordinate file. Navigate to the directory (testing/testMaps) containing the map file 'ecoli_core_map.txt' and then execute the following command:

```
>> map = readCbMap('ecoli_core_map.txt');
```

Display a metabolic map.

```
>> changeCbMapOutput('matlab');
>> drawCbMap(map);
```

The following example illustrates one of the many ways to change the appearance of a map. Colors are based on the RGB style. To change all of the nodes to black and the edges to red, first create an $n \times 3$ matrix, where n is the number of nodes in the map and each row is the RGB for black ([0,0,0]).

```
>> node_colors = repmat([0,0,0],size(map.molName,1),1);
```

Next, create an $n \times 3$ matrix, where n is the number of edges in the map and each row is the RGB for red ([1,0,0]).

```
>> edge_colors = repmat([1,0,0],size(map.connectionName,1),1);
```

Next, create a map options structure. The first argument is either an empty matrix or a previously created map options structure.

```
>> options = setMapOptions([], map, 'nodeColor', node_colors, 'edgeColor', edge_colors);
>> drawCbMap(map, options);
```

Save a metabolic map.

```
>> changeCbMapOutput('svg')
>> drawCbMap(map);
```

The file 'target.svg' will be saved in the working directory.

Optimal flux distributions and growth rates for *E. coli* core model

To read in the *Escherichia coli* (*E. coli*) core model and predict a flux distribution for optimal growth, navigate to the directory (testing/testMaps) containing the *E. coli* core model and map file, and then execute the following functions:

```
>> model = readCbModel('ecoli_core_model.xml');
>> map = readCbMap('ecoli_core_map.txt');
>> changeCbMapOutput('svg');
>> solution = optimizeCbModel(model);
```

The expected optimal biomass flux (solution.f) is ~0.87.

```
>> drawFlux(map, model, solution.x, [], 'FileName', 'EcoreOptFlux1.svg');
```

The drawFlux function call generates an .svg file named EcoreOptFlux1.svg in the working directory. The reactions are color coded using a linear scale from cyan (corresponding to a flux of −29.17) to magenta (corresponding to a flux of 45.51).

To more easily extract data from the map, change the width of the reaction arrows corresponding to reactions carrying zero flux to 1 point. In addition, set the lower and upper bounds to −15 and 15, respectively.

```
>> drawFlux(map, model, solution.x, [], 'ZeroFluxWidth', 1, 'lb', −15, 'ub', 15, 'FileName', 'EcoreOptFlux2.svg');
```

An .svg file named EcoreOptFlux2.svg should be saved in the working directory, with reactions color coded from cyan (flux of −15 or less) to magenta (flux of 15 or greater); reactions carrying zero flux have their corresponding arrows narrowed (**Fig. 3**).

Parsimonious FBA categorization of genes for *E. coli* growth on acetate

To perform pFBA on the *E. coli* core model growing in an acetate minimal medium and to plot the results, navigate to the directory (testing/testMaps) containing the *E. coli* core model and execute the following commands:

```
>> map = readCbMap('ecoli_core_map.txt');
>> model = readCbModel('ecoli_core_model.xml');
```

Remove glucose from the minimal medium.

```
>> model = changeRxnBounds(model,'EX_glc(e)',0,'l');
```

Add acetate to the minimal medium.

```
>> model = changeRxnBounds(model,'EX_ac(e)',-10,'l');
>> [pFBAGeneClasses pFBARxn] = pFBA(model, 'map', map, 'mapOutName', 'Ecore_pFBA_ac.svg');
```

A reaction map, with reactions color coded according to the reaction class, will be saved as 'Ecore_pFBA_ac.svg' in the working directory. Inspecting the map will show that pyruvate kinase (PYK) is classified by pFBA as enzymatically less efficient. This is because its use does not reduce the computed growth rate, but it increases the amount of flux through the network. Interestingly, it has been reported that this enzyme is downregulated in wild-type *E. coli* when grown on acetate minimal medium[69]. Simulation time is ~20 s with the Gurobi LP solver.

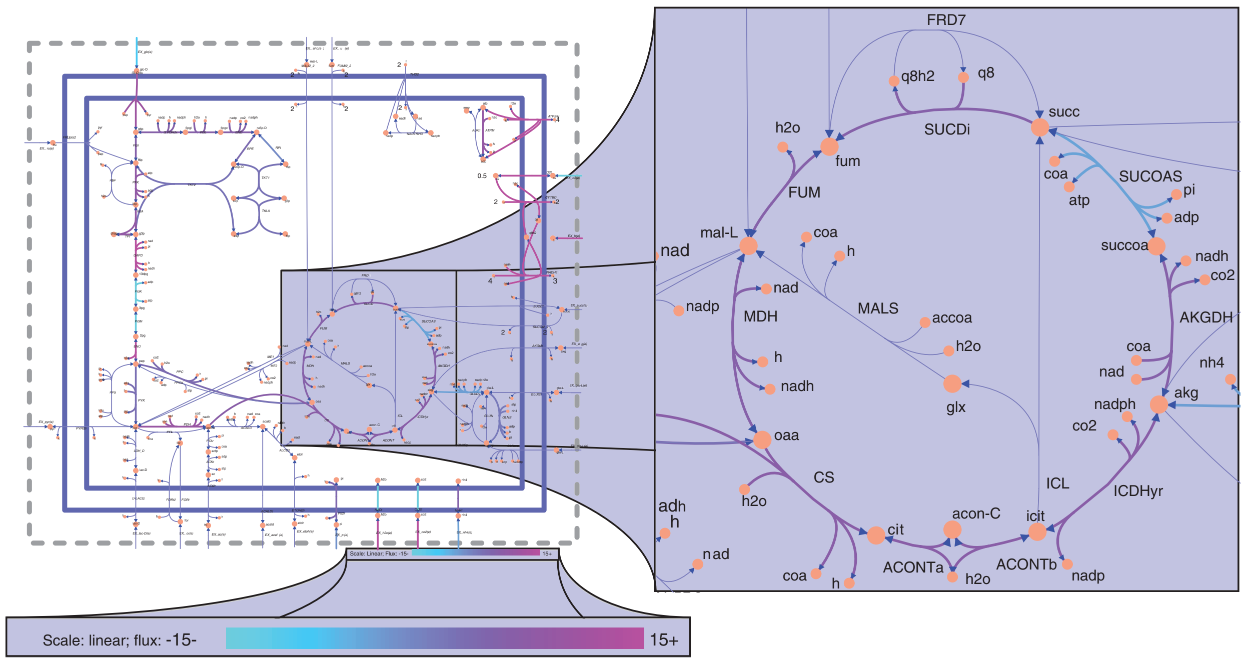


Figure 3 | Flux balance analysis of *E. coli* core model. (Left) Full *E. coli* core map. (Right) Zoom in on the optimal flux distribution map of the citric acid cycle. (Bottom) Zoom in on the flux color scale. Reactions are colored according to a scale of cyan (flux of 15 mmol per gDW per h or greater in the reverse direction) to magenta (flux of 15 mmol per gDW per h) or greater in the forward direction). Reactions carrying zero flux have their corresponding arrows narrowed.

FVA of *E. coli* core model
To perform FVA for the *E. coli* core model under glucose-limited aerobic growth conditions with a minimum cellular growth of 90% of optimal, navigate to the directory (testing/testMaps) containing the *E. coli* core model and execute the following commands:

```
>> model = readCbModel('ecoli_core_model.xml');
>> [minFlux, maxFlux] = fluxVariability(model,90);
```
Simulation time is ~2 s with the Gurobi LP solver.
```
>> map = readCbMap('ecoli_core_map.txt');
>> changeCbMapOutput('svg');
>> drawFluxVariability(map, model, minFlux, maxFlux, [], 'fileName', 'EcoreFluxVariability.svg');
```
A reaction map, with reactions color coded according to the flux directionality that it can carry, will be saved to the file 'EcoreFluxVariability.svg' in the current working directory (**Fig. 4**). Bidirectional reversible reactions are colored green. Unidirectional reversible reactions that carry flux in the forward direction are colored magenta. Unidirectional reversible reactions that carry flux only in the reverse direction are colored cyan. Irreversible fluxes are colored blue. Unidirectional fluxes have enlarged arrowheads in the direction of the flux.

Sampling of the solution space of *E. coli* core model for growth in aerobic versus anaerobic conditions
To read in the *E. coli* core model and to sample its solution space under glucose minimal media and aerobic conditions with 200 points for 2 min, navigate to the directory (testing/testMaps) containing the *E. coli* core model and execute the following commands:

```
>> model_aerobic = readCbModel('ecoli_core_model.xml');
>> sampleStruct_aerobic = gpSampler(model_aerobic,200,[],120);
```
Simulation time is ~120 s with the Gurobi LP solver.
```
>> model_anaerobic = changeRxnBounds(model_aerobic,'EX_o2(e)',0,'l');
>> sampleStruct_anaerobic = gpSampler(model_anaerobic,200,[],120);
```
Simulation time is ~120 s with the Gurobi LP solver. Sampling results will be returned in the two structures sampleStruct_aerobic and sampleStruct_anaerobic within the field points.

Visualize sampling results for a set of reactions.
```
>> rxnList = {'PGI', 'PFK', 'FBP', 'FBA', 'TPI', 'GAPD', 'PGK', 'PGM', 'ENO', 'PYK'};
>> plotSampleHist(rxnList, {sampleStruct_aerobic.points, sampleStruct_anaerobic.points }, {model_aerobic, model_anaerobic},[],[2,5]);
```

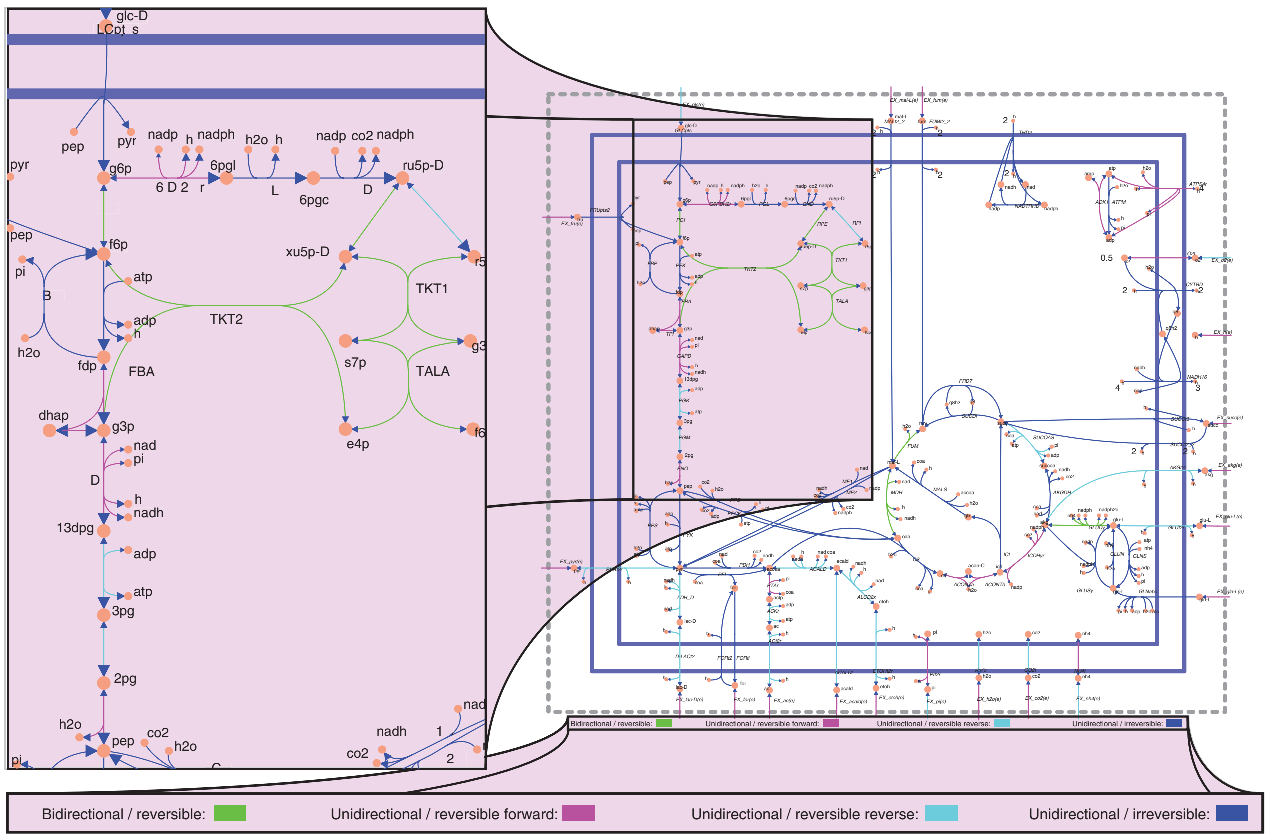


Figure 4 | Flux variability analysis of *E. coli*. (Right) Reaction map of *E. coli* core model. (Left) Flux variability analysis of part of glycolysis and pentose phosphate pathway in the *E. coli* core model when growth rate is constrained to 90% of optimal. Bidirectional reversible reactions are colored green. Unidirectional reversible reactions that carry flux in the forward direction are colored magenta. Unidirectional reversible reactions that carry flux only in the reverse direction are colored cyan. Irreversible fluxes are colored blue. Unidirectional fluxes have enlarged arrowheads in the direction of the flux.

A MATLAB figure will also be generated showing the histograms for glycolysis with aerobic in blue and anaerobic in green (**Fig. 5**).

Identifying gaps in the metabolic network

To find gaps in the Ec_iJR904 model using the gapFind function, navigate to the directory (testing/testReadWrite) containing the Ec_iJR904.xml and execute the following commands:

```
>> model = readCbModel('Ec_iJR904.xml');
>> exchangeRxns = model.rxns(findExcRxns(model));
```

Set the lower bound for all reversible reactions to -1×10^6.

```
>> model = changeRxnBounds(model, model.rxns(logical(model.rev)), -1e6, 'l');
```

Set the upper bound for all reactions to 1×10^6.

```
>> model = changeRxnBounds(model, model.rxns, 1e6, 'u');
```

Set the lower bound for all exchange reactions to −1 to allow for uptake.

```
>> model = changeRxnBounds(model, exchangeRxns, -1, 'l');
>> [allGaps, rootGaps, downstreamGaps] = gapFind(model);
```

Using the Gurobi MILP solver (simulation time 1.5 s), there are 64 metabolites identified as gaps: 28 root gaps and 36 downstream gaps; using the GLPK solver, only 20 of the 36 downstream gaps are identified.

Filling gaps using growthExpMatch. Remove the PGK reaction from the *E. coli* core model and use growthExpMatch to propose candidate reactions required to allow growth on glucose. Navigate to the directory (testing/testGrowthExpMatch) containing the *E. coli* core model and the universal reaction database, and execute the following commands:

```
>> model = readCbModel('ecoli_core_model.xml');
>> modelKO = removeRxns(model,{'PGK'});
>> KEGGFilename = '2010_07_30_KEGG_reaction.lst';
>> load('Dictionary.mat');
>> growthExpMatch(modelKO, KEGGFilename, '[c]', 5, dictionary,'GEMLog.txt');
```

The PGK reaction is removed from the *E. coli* core model, thus removing the ability of the model to produce biomass from glucose. Updated versions of the KEGG reaction list should be downloaded from the KEGG website (http://www.genome.jp/kegg)[67,68]. The resulting GEMLog file should contain five solutions (**Table 3**); please note that if no solutions are found, then the log file will not be generated. The first solution R01512 corresponds to the PGK reaction that was removed previously. The remaining four solutions are alternate reaction sets that, when added, allow the model to grow on glucose. With the Gurobi MILP solver, simulation time is ~2×10^3 s.

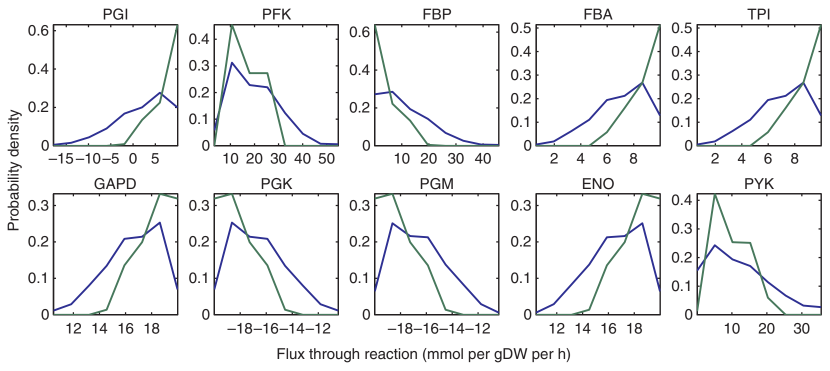


Figure 5 | Sampling histogram of glycolysis, using the *E. coli* core model under aerobic and anaerobic glucose minimal medium conditions. For growth in aerobic (blue) versus anaerobic (green) medium, there is a large shift in the probable flux through many of the reactions. In general, the range of flux probabilities for each reaction became more constrained. Phosphoglucose isomerase (PGI) switched from being able to carry flux in either direction with aerobic conditions to only carrying flux in the forward direction with anaerobic conditions.

Optimize product secretion using the *E. coli* core model

Set up model for strain design. Navigate to the directory (testing/testMaps) containing the *E. coli* core model and execute the following commands.

```
>> model = readCbModel('ecoli_core_model.xml');
```

Adjust the minimal medium composition to be anaerobic and contain a supply of glucose (20 mmol per gDW per h).

```
>> model = changeRxnBounds(model, {'EX_o2(e)', 'EX_glc(e)'}, [0, –20], 'l');
```

Build a list of candidate reactions for deletion to optimize product formation. It is wise to exclude exchange and transport reactions, and biomass and ATP maintenance requirements.

```
>> selectedRxns = {model.rxns{ [1, 3:5, 7:8, 10, 12, 15:16, 18, 40:41, 44, 46, 48:49, 51, 53:55, 57, 59:62, 64:68, 71:77, 79:83, 85:86, 89:95]}};
```

OptKnock analysis of model. To optimize for lactate secretion with five deletions or less using the OptKnock method:

```
>> options.targetRxn = 'EX_lac-D(e)';
>> options.vMax = 1000;
>> options.numDel = 5;
>> options.numDelSense = 'L';
```

TABLE 3 | GrowthExpMatch gap-filling solutions.

#	KEGG ID	Reaction name	Reaction formula
1	'R01512_b'	Phosphoglycerate kinase	ATP + 3-phospho-D-glycerate ⇔ ADP + 3-phospho-D-glyceroyl phosphate
2	'R02188_f'	3-Phospho-D-glyceroyl-phosphate: polyphosphate phosphotransferase	3-Phospho-D-glyceroyl phosphate + (phosphate)n ⇔ 3-phospho-D-glycerate + (phosphate)n
3	'R01515_f'	Acylphosphatase	3-Phospho-D-glyceroyl phosphate + H_2O ⇔ 3-phospho-D-glycerate + orthophosphate
4	'R00761_f'	Fructose-6-phosphate phosphoketolase	D-Fructose 6-phosphate + orthophosphate ⇔ acetyl phosphate + D-erythrose 4-phosphate + H_2O
5	'R00024_f' 'R01523_f'	Ribulose-bisphosphate carboxylase Phosphoribulokinase	D-Ribulose 1,5-bisphosphate + CO_2 + H_2O ⇔ 2,3-Phospho-D-glycerate ATP + D-ribulose 5-phosphate ⇔ ADP + D-ribulose 1,5-bisphosphate

Solutions from five iterations of growthExpMatch on a PGK knockout growing on glucose using the *E. coli* core model. The first solution returned is the knocked-out reaction. Solutions from iterations 2, 3 and 5 are reactions to use 3-phospho-D-glycerol phosphate while the solution from iteration 4 bypasses the gap by converting D-fructose 6-phosphate to D-erythrose 4-phosphate and acetyl phosphate.

TABLE 4 | Expected results from OptKnock and GDLS optimizations for lactate, succinate and pyruvate production growing on glucose.

	OptKnock			GDLS		
Target	Lactate	Succinate	Pyruvate	Lactate	Succinate	Pyruvate
Knockout list	ALCD2x, FUM, GLUDY, ME2, PYK	ALCD2x, GLUDy, LDH_D, PFL, THD2	ACALD, LDH_D, PTAr	ACALD, FRD7, GLUDy, ME2, PYK	ALCD2x, GLUDy, LDH_D, PFL, THD2	ACALD, LDH_D, PTAr
Product (mmol per gDW per h)	37.7	20.2–26.1	18.9	37.7	20.2–26.1	18.9
Biomass (mmol per gDW per h)	0.142	0.120	0.151	0.142	0.120	0.151
Computational time (s)	28.1	24.5	5.6	4.9	2.9	1.8

The solutions for succinate and pyruvate are the same for both methods. The lactate solutions vary by two reactions; however, both resulting models have the same production and growth rates.

```
>> constrOpt.rxnList = {'Biomass_Ecoli_core_N(w/GAM)-Nmet2', 'ATPM'};
>> constrOpt.values = [0.05, 8.39];
>> constrOpt.sense = 'GE';
>> optKnockSol = OptKnock(model, selectedRxns, options, constrOpt);
```

The resulting knockout list (optKnockSol.rxnList) is alcohol dehydrogenase, fumarase, glutamate dehydrogenase, malic enzyme (NADP) and pyruvate kinase (**Table 4**). The resulting knockout predicted growth rate of ~0.142 and product excretion rate of ~37.7. The computational time required for this simulation is ~4 s with the Gurobi MILP solver.

Strain design using GDLS. Execute the following commands to optimize for the lactate secretion using the GDLS algorithm.

```
>> [gdlsSolution, bilevelMILPproblem, gdlsSolutionStructs] = GDLS(model, 'EX_lac-D(e)', 'minGrowth', 0.05, 'selectedRxns', selectedRxns, 'maxKO', 5, 'nbhdsz', 3);
```

The resulting knockout list (gdlsSolution.KOs) is acetaldehyde dehydrogenase, fumarate reductase, glutamate dehydrogenase, phosphotransacetylase and NAD(P) transhydrogenase (**Table 4**). The resulting knockout predicted a growth rate of ~0.14 and a product excretion rate of ~37.7. The computational time required for this simulation is ~2 s with the Gurobi LP solver.

Both methods were also used to optimize for succinate product with a maximum of five knockouts, and pyruvate with a maximum of three knockouts. For succinate and pyruvate, the list of reactions to knock out was the same for both OptKnock and GDLS; however, two reactions were different when the target product was lactate (**Table 4**). For lactate, OptKnock chose alcohol dehydrogenase and fumarase, whereas GDLS chose acetaldehyde dehydrogenase and fumarate reductase. However, both result in the same optimal flux distribution.

Build a draft tissue-specific human macrophage model from the global human metabolic network and omics data
Navigate to testing/testTissueModel. To save time, we have provided a MAT file (testTissueModel.mat) that contains the global human metabolic network *model* and a formatted *expressionData* structure. The *model* is the version of the human metabolic network reconstruction[70] that was used to create an alveolar macrophage model[14] using expression data from Kazeros *et al.*[71]

```
>> load('testTissueModel.mat')
```

Build a draft model with GIMME. The GIMME algorithm retains reactions from Recon 1 that are orphans or are present in the high-throughput data. The reactions with no detected expression are minimized and those not required to retain flux through the objective reaction are removed.

```
>> [tissueModel,Rxns] = createTissueSpecificModel(model,expressionData);
```

Where tissueModel is the GIMME algorithm-derived draft model; and Rxns is a structure with lists of all the reactions. The reactions fall into the following categories: Expressed—1,769 potentially active reactions based on transcriptome data; UnExpressed—497 reactions without requisite gene products based on transcriptome data; Unknown—41 reactions unable to be predicted by transcriptome data; Upregulated—52 UnExpressed reactions added back into model; Downregulated—0 Expressed reactions removed from model; and UnknownIncluded—1,476 orphan reactions included. The calculations should take ~50 s with Gurobi LP solver for GIMME.

Note: Supplementary information is available in the HTML version of this article.

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