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Creation and analysis of biochemical constraint-based models: the COBRA Toolbox v3.0

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Abstract

COnstraint-Based Reconstruction and Analysis (COBRA) provides a molecular mechanistic framework for integrative analysis of experimental molecular systems biology data and quantitative prediction of physicochemically and biochemically feasible phenotypic states. The COBRA Toolbox is a comprehensive desktop software suite of interoperable COBRA methods. It has found widespread applications in biology, biomedicine, and biotechnology because its functions can be flexibly combined to implement tailored COBRA protocols for any biochemical network. This protocol is an update to the COBRA Toolbox 1.0 and 2.0. Version 3.0 includes new methods for quality controlled reconstruction, modelling, topological analysis, strain and experimental design, network visualisation as well as network integration of chemoin-formatic, metabolomic, transcriptomic, proteomic, and thermochemical data. New multi-lingual code integration also enables an expansion in COBRA application scope via high-precision, high-performance, and nonlinear numerical optimisation solvers for multi-scale, multi-cellular and reaction kinetic modelling, respectively. This protocol overviews all of these new features and can be adapted to generate and analyse constraint-based models in a wide variety of scenarios. The COBRA Toolbox 3.0 provides an unparalleled depth of constraint-based reconstruction and analysis methods.

Keywords

Metabolic models; metabolic reconstruction; metabolic engineering; gap filling; strain engineering; omics; data integration; metabolomics; transcriptomics; constraint-based modelling; computational biology; bioinformatics; biochemistry; human metabolism; microbiome analysis

INTRODUCTION

Development of the protocol

Constraint-based reconstruction and analysis (COBRA[1]) is a mechanistic integrative analysis framework that is applicable to any biochemical system with prior mechanistic information, including where mechanistic information is incomplete. The overall approach is to mechanistically represent the relationship between genotype and phenotype by mathematically and computationally modelling the constraints that are imposed on the

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phenotype of a biochemical system by physicochemical laws, genetics, and the environment[2] (see Figure 1). This protocol updates and extends previous protocols on the COBRA Toolbox versions 1.0[3] and 2.0[4]. It provides an introduction into the practical application of many of the novel COBRA methods developed in recent years.

Early in the development of the COBRA framework, the need for ease of reproducibility and demand for reuse of COBRA methods were recognised. This necessity led to the COBRA Toolbox version 1.0[3], an open source software package running in the MATLAB environment, which facilitated quantitative prediction of metabolic phenotypes using a selection of the COBRA methods available at the time. With the expansion of the COBRA community and the growing phylogeny of COBRA methods, the need was recognised for the amalgamation and transparent dissemination of COBRA methods. This demand led to the COBRA Toolbox version 2.0[4], with an enhanced range of methods to simulate, analyse, and predict a variety of phenotypes using genome-scale metabolic reconstructions. Since then, the increasing functional scope and size of biochemical network reconstructions, as well as the increasing breadth of physicochemical and biological constraints that are represented within constraint-based models, naturally result in the development of a broad arbour of new COBRA methods[5].

The present protocol overviews the main novel developments within version 3.0 of the COBRA Toolbox (see Table 1), especially the expansion of functionality to cover new biochemical network reconstruction and modelling methods. In particular, this protocol includes the input and output of new standards for sharing reconstructions and models, an extended suite of supported general purpose optimisation solvers, new optimisation solvers developed especially for constraint-based modelling problems, enhanced functionality in the areas of computational efficiency and high precision computing, numerical characterisation of reconstructions, conversion of reconstructions into various forms of constraint-based models, comprehensive support for flux balance analysis and its variants, integration with omics data, uniform sampling of high dimensional models, atomic resolution of metabolic reconstructions via molecular structures, estimation and application of thermodynamic constraints, visualisation of metabolic networks, and genome-scale kinetic modelling.

This protocol consists of a set of methods that are introduced in sequence but can be combined in a multitude of ways. The overall purpose is to enable the user to generate a biologically relevant, high-quality model that enables novel predictions and hypotheses generation. Therefore, we implement and enforce standards in reconstruction and simulation that have been developed by the COBRA community over the past two decades. All explanations of a method are also accompanied by explicit computational commands.

First, we explain how to initialise and verify the installation of the COBRA Toolbox in MATLAB (Math-works, Inc.). The main options to import and explore the content of a biochemical network reconstruction are introduced. For completeness, a brief summary of methods for manual and algorithmic reconstruction refinement are provided, with reference to the established reconstruction protocol[6]. We also explain how to characterise the numerical properties of a reconstruction, especially with respect to detection of a reconstruction requiring a multi-scale numerical optimisation solver. We explain how to

semi-automatically convert a reconstruction into a constraint-based model suitable for flux balance analysis. This is followed by an extensive explanation of how to carry out flux balance analysis and its variants. The procedure to fill gaps in a reconstruction, due to missing reactions, is also explained.

We provide an overview of the main methods to integrate metabolomic, transcriptomic, proteomic, and thermochemical data to generate context-specific, constraint-based models. Various methods are explained for the addition of biological constraints to a constraint-based model. We then explain how to test the chemical and biochemical fidelity of the model. Now that a high-quality model is generated, we explain how to interrogate the discrete geometry of its stoichiometric subspaces, how to efficiently measure the variability associated with the prediction of steady state reaction rate using flux variability analysis, and how to uniformly sample steady-state fluxes. We introduce various approaches for prospective uses of a constraint-based model, such as strain and experimental design.

We explain how to atomically resolve a metabolic reconstruction by connecting it with molecular species structures and how to use cheminformatic algorithms for atom mapping and identification of conserved moieties. Using molecular structures for each metabolite, and established thermochemical data, we estimate the transformed Gibbs energy of each subcellular compartment specific reaction in a model of human metabolism in order to thermodynamically constrain reaction directionality and constrain the set of feasible kinetic parameters. Sampled kinetic parameters are then used for variational kinetic modelling, in an illustration of the utility of recently published algorithms for genome-scale kinetic modelling. We also explain how to visualise predicted phenotypic states using a recently developed approach for metabolic network visualisation. We conclude with an explanation of how to engage with the community of COBRA developers, as well as contribute code to the COBRA Toolbox with MATLAB.devTools, a newly developed piece of software for community contribution of COBRA methods to the COBRA Toolbox.

All documentation and code is released as part of the *openCOBRA* project (https://github.com/opencobra/cobratoolbox). Where reading the extensive documentation associated with the COBRA Toolbox does not suffice, we describe the procedure for effectively engaging with the community via a dedicated online forum (https://groups.google.com/forum/#!forum/cobra-toolbox). Taken together, the COBRA Toolbox 3.0 provides an unparalleled depth of interoperable COBRA methods and a proof-of-concept that knowledge integration and collaboration by large numbers of scientists can lead to cooperative advances impossible to achieve by a single scientist or research group alone[7].

Applications of COBRA methods

Constraint-based modelling of biochemical networks is broadly applicable to a range of biological, biomedical, and biotechnological research questions[8]. Fundamentally, this broad applicability arises from the common phylogenetic tree, shared by all living organisms, that manifests in a set of shared mathematical properties that are common to biochemical networks in normal, diseased, wild-type, or mutant biochemical networks. Therefore, a COBRA method developed primarily for use in one scenario can usually be quickly adapted for use in a variety of related scenarios. Often, this adaptation retains the mathematical

properties of the optimisation problem underlying the original constraint-based modelling method. By adapting the input data and interpreting the output results in a different way, the same method can be used to address a different research question.

Biotechnological applications of constraint-based modelling include the development of sustainable approaches for chemical[9] and biopharmaceutical production[10, 11]. Among these applications is the computational design of new microbial strains for production of bioenergy feedstocks from non-food plants, such as microbes capable of deconstructing biomass into their sugar subunits and synthesising biofuels, either from cellulosic biomass or through direct photosynthetic capture of carbon dioxide.

Another prominent biotechnological application is the analysis of interactions between organisms that form biological communities and their surrounding environments, with a view toward utilisation of such communities for bioremediation[12] or nutritional support of non-food plants for bioenergy feedstocks. Biomedical applications of constraint-based modelling include the prediction of the phenotypic consequences of single nucleotide polymorphisms[13], drug targets[14], enzyme deficiencies[15–18], as well as side and off-target effects of drugs[19–21]. COBRA has also been applied to generate and analyse normal and diseased models of human metabolism[17, 22–25], including organ-specific models[26–28], multi-organ-models[29, 30], and personalised models[31–33]. Constraint-based modelling has also been applied to understanding of the biochemical pathways that interlink diet, gut microbial composition, and human health[34–38].

Key features and comparisons

Besides the COBRA Toolbox, constraint-based reconstruction and analysis can be carried out with a variety of software tools. In 2012, Lakshmanan et al.[39] made a comprehensive, comparative evaluation of the usability, functionality, graphical representation and inter-operability of the tools available for flux balance analysis. Each of these evaluation criteria is still valid when comparing the current version of the COBRA Toolbox with other software with constraint-based modelling capabilities. The rapid development of novel constraint-based modelling algorithms requires continuity of software development. Short term investment in new COBRA modelling software applications has led to a plethora of COBRA modelling applications[39]. Each usually provides some unique capability initially, but many have become antiquated due to lack of maintenance, failure to upgrade, or failure to support new standards in model exchange formats (http://sbml.org/Documents/Specifications). Therefore, we also restrict our comparison to software in active development (see Table 2).

Each software tool for constraint-based modelling has varying degrees of dependency on other software. Web-based applications exist for the implementation of a limited number of standard constraint-based modelling methods. Their only local dependency is on a web browser. The COBRA Toolbox depends on MATLAB (Mathworks Inc.), a commercially distributed, general-purpose computational tool. MATLAB is a multi-paradigm programming language and numerical computing environment that allows matrix manipulations, plotting of functions and data, implementation of algorithms, creation of user interfaces, and interfacing with programs written in other languages, including C, C++, C#, Java, Fortran, and Python. All software tools for constraint-based modelling also depend on

at least one numerical optimisation solver. The most robust and efficient numerical optimisation solvers for standard problems are distributed commercially, but often with free licences available for academic use, e.g., Gurobi Optimizer (http://www.gurobi.com). Stand-alone constraint-based modelling software tools also exist and their dependency on a numerical optimisation solver is typically satisfied by GLPK (https://gnu.org/software/glpk), an open-source linear optimisation solver.

It is sometimes perceived that there is a commercial advantage to depending only on open-source software. However, there are also commercial costs associated with dependency on open-source software. That is, in the form of increased computation times as well as increased time required to install, maintain and upgrade open-source software dependencies. This is an important consideration for any research group whose primary focus is on biological, biomedical, or biotechnological applications, rather than on software development. The COBRA Toolbox 3.0 strikes a balance by depending on closed-source, general purpose, commercial computational tools, yet all COBRA code is distributed and developed in an open-source environment (https://github.com/opencobra/cobratoolbox).

The availability of comprehensive documentation is an important feature in the usability of any modelling software. Therefore, a dedicated effort has been made to ensure that all functions in the COBRA Toolbox 3.0 are comprehensively and consistently documented. Moreover, we also provide a new suite of more than 35 tutorials (https://opencobra.github.io/cobratoolbox/latest/tutorials) to enable beginners, as well as intermediate and advanced users to practise a wide variety of COBRA methods. Each tutorial is presented in a variety of formats, including as a MATLAB live script, which is an interactive document, or *narrative*, (https://mathworks.com/help/matlab/matlab_prog/what-is-a-live-script.html) that combines MATLAB code with embedded output, formatted text, equations, and images in a single environment viewable with the MATLAB Live Editor (version R2016a or later). MATLAB live scripts are similar in functionality to Mathematica Notebooks (Wolfram Inc.) and Jupyter Notebooks (https://jupyter.org). The latter support interactive data science and scientific computing for more than 40 programming languages. To date, only the COBRA Toolbox 3.0, COBRApy[40], KBase[41], and COBRA.jl[42] offer access to constraint-based modelling algorithms via narratives.

KBase is a collaborative, open environment for systems biology of plants, microbes and their communities[41]. It also has a suite of analysis tools and data that support the reconstruction, prediction, and design of metabolic models in microbes and plants. These tools are tailored toward the optimisation of microbial biofuel production, the identification of minimal media conditions under which that fuel is generated, and predict soil amendments that improve the productivity of plant bioenergy feedstocks. In our view, KBase is currently the tool of choice for the automatic generation of draft microbial metabolic networks, which can then be imported into the COBRA Toolbox for further semi-automated refinement, which has recently successfully been completed for a suite of gut microbial organisms[38]. However, KBase[41] currently offers a modest depth of constraint-based modelling algorithms.

MetaFlux[43] is a web-based tool for the generation of network reconstructions directly from pathway and genome databases, proposing network refinements to generate functional flux balance models from reconstructions, predict steady-state reaction rates with flux balance analysis and interpret predictions in a graphical network visualisation. MetaFlux is tightly integrated within the PathwayTools[44] environment, which provides a broad selection of genome, metabolic and regulatory informatics tools. As such, PathwayTools provides breadth in bioinformatics and computational biology, while the COBRA Toolbox 3.0 provides depth in constraint-based modelling, without providing, for example, any genome informatics tools. Although an expert can locally install a PathwayTools environment, the functionality is closed source and only accessible via an application programming interface. This approach does not permit the level of repurposing possible with open-source software. As recognised in the computational biology community[45], open-source development and distribution is scientifically important for tractable reproducibility of results as well as reuse and repurposing of code[46].

Lakshmanan et al.[39] consider the availability of a graphical user interface to be an important feature in the usability of modelling software. For example, SurreyFBA[47] provides a command line tool and graphical user interface for constraint-based modelling of genome-scale metabolic reaction networks. The time lag between the development of a new modelling method and its availability via a graphical user interface necessarily means that graphically driven COBRA tools permit a limited depth of novel constraint-based modelling methods. While MATLAB provides a generic graphical user interface, the COBRA Toolbox is controlled either by scripts or narratives, rather than graphically. Exceptions include the input of manually-curated data during network reconstruction[48], the assimilation of genome-scale metabolic reconstructions[49], and the visualisation of simulation results in biochemical network maps[50] via specialised network visualisation software[51].

Due to the relative simplicity of the MATLAB programming language, new COBRA Toolbox users, including those without software development experience, can rapidly become familiar with the basics of constraint-based modelling. This initial learning effort is worth it for the flexibility it opens up, especially considering the broad array of constraint-based modelling methods now available within the COBRA Toolbox 3.0. Although it should be technically possible to generate a computational specification of the point-and-click analysis steps that are required to generate results using a graphical user interface, to our knowledge, none of the graphically-driven modelling tools in Table 2 offers this facility. Such a specification would be required for another scientist to reproduce the same results using the same tool. This weakness limits the ability to reproduce analytical results, as verbal specification is not sufficient for reproducibility[46].

Each language-specific COBRA implementation has its benefits and drawbacks, which are mainly associated with the programming language itself. PySCeS-CBM[52] and COBRApy[40] both provide support for a set of COBRA methods implemented in the Python programming language. Python is a multi-paradigm, interpreted programming language for general-purpose programming. It has a broad development community and a wide range of open-source libraries, especially in bioinformatics. As such, it is well suited for the amalgamation and management of heterogeneous experimental data. At present, the COBRA software

tools in Python provide access to standard COBRA methods. In COBRApy[40], this functionality can be extended by using Python to invoke MATLAB and use the COBRA Toolbox. Achieving such interoperability between COBRA software implemented in different programming languages and developed together by a united open source community is the primary objective of the openCOBRA project (https://opencobra.github.io).

Sybil[53] is an open-source, object-oriented software library that implements a limited set of standard constraint-based modelling algorithms in the programming language R, which is a free, platform independent environment for statistical computing and graphics. Sybil is available for download from the comprehensive R archive network (CRAN), but does not follow an open-source development model. The COBRA Toolbox is primarily implemented in MATLAB, a proprietary, multi-paradigm, programming language which is interpreted for execution rather than compiled prior to execution. As such, MATLAB code typically runs slower than compiled code, but the main advantage is the ability to rapidly and flexibly implement sophisticated numerical computations by leveraging the extensive libraries for general-purpose numerical computing, supplied commercially within MATLAB (Mathworks Inc.), and distributed freely by the community (https://mathworks.com/matlabcentral).

For the application of computationally-demanding constraint-based modelling methods to high-dimensional or high-precision constraint-based models, the COBRA Toolbox 3.0 comes with an array of integrated, pre-compiled extensions and interfaces that employ complementary programming languages and tools. These include a quadruple precision Fortran 77 optimisation solver implementation for constraint-based modelling of multi-scale biochemical networks[54], and a high-level, high-performance, open-source implementation of flux balance analysis in Julia[42]. The latter is tailored to solve multiple flux balance analyses on a subset or all the reactions of large- and huge-scale networks, on any number of threads or nodes. To enumerate elementary modes or minimal cut-sets, we provide an interface to CellNetAnalyzer[55, 56] (https://www2.mpi-magdeburg.mpg.de/projects/cna/cna.html), which excels at computationally-demanding, enumerative, discrete geometry calculations of relevance to biochemical networks. In addition, we included an updated implementation of the genetic minimal cut-sets approach[57], which extends the concept of minimal cut-sets to gene knockout interventions.

In summary, the COBRA Toolbox 3.0 provides an unparalleled depth of constraint-based reconstruction and analysis methods, has a highly active and supportive open-source development community, is accompanied by extensive documentation and narrative tutorials, it leverages the most comprehensive library for numerical computing, and it is distributed with extensive interoperability with a range of complementary programming languages that exploit their particular strengths to realise specialised constraint-based modelling methods. A list of the main COBRA methods now available in the COBRA Toolbox is given in Table 1. Moreover, all of this functionality is provided within one accessible software environment.

Experimental Design

The COBRA Toolbox 3.0 is designed for flexible adaptation into customised pipelines for constraint-based reconstruction and analysis in a wide range of biological, biochemical, or biotechnological scenarios, from single organisms to communities of organisms. To become proficient in adapting the COBRA Toolbox to generate a protocol specific to one's situation, it is wise to first familiarise oneself with the principles of constraint-based modelling. This can best be achieved by studying the educational material already available. The textbook *Systems Biology: Constraint-based Reconstruction and Analysis*[1] is an ideal place to start. It is accompanied by a set of lecture videos that accompany each chapter http://systemsbiology.ucsd.edu/Publications/Books/SB1–2LectureSlides. The textbook *Optimization Methods in Metabolic Networks*[58] provides the fundamentals of mathematical optimisation and its application in the context of metabolic network analysis. A study of this educational material will accelerate one's ability to utilise any software application dedicated to COBRA.

Once one is cognisant of the conceptual basis of COBRA, one can then proceed with this protocol, which summarises a subset of the key methods that are available within the COBRA Toolbox. To adapt this protocol to one's situation, users can combine the COBRA methods implemented within the COBRA Toolbox in numerous ways. The adaption of this protocol to one's situation may require the development of new customised MATLAB scripts that combine existing methods in a new way. Due to the aforementioned benefits of narratives, the first choice should be to implement these customised scripts in the form of MATLAB live scripts. To get started, the existing tutorial narratives, described in Table 1, can be repurposed as templates for new analysis pipelines. Narrative figures and tables can then be generated from raw data and used within the main text of scientific articles and converted into supplementary material to enable full reproducibility of computational results. The narratives specific to individual scientific articles can also be shared with peers within https://github.com/opencobra/cobratoolbox/tree/master/papers.

New tutorials can be shared with the COBRA community: https://git.io/COBRA.tutorials. Depending on one's level of experience, or the novelty of an analysis, the adaptation of this protocol to a particular situation may require the adaption of existing COBRA methods, or development of new COBRA methods, or both.

Software architecture of the COBRA Toolbox 3.0—The source code of the COBRA Toolbox (https://github.com/opencobra/cobratoolbox/tree/master/src) is divided into several top-level folders, which either mimic the main classes of COBRA methods (*reconstruction*, *dataIntegration*, *analysis*, *visualisation*, *design*) or contain the basic functions (*base*) available for use within many COBRA methods. For example, the input or output of reconstructions and models in various formats as well as all the interfaces to optimisation solvers is contained within the *base* folder. The *reconstruction* folder contains all of the methods associated with the reconstruction and refinement of a biochemical network to match experimental data, as well as the conversion of a reconstruction into various forms of constraint-based models (see Table 3 for a description of the main fields of a COBRA model). The *dataIntegration* folder contains the methods for integration of metabolomic,

transcriptomic, proteomic, and thermodynamic data with a reconstruction or model. The *analysis* folder contains all of the methods for interrogation of the properties of a reconstruction or model, and combinations thereof, as well as the prediction of biochemical network states using constraint-based models. The *visualisation* folder contains all of the methods for the visualisation of predictions within a biochemical network context, using various biochemical cartography tools that interoperate with the COBRA Toolbox. The *design* folder contains new strain design methods and a new modelling language interface to GAMS (General Algebraic Modeling System), a high-level modeling system for mathematical optimisation[59].

Open-source software development with the COBRA Toolbox—Understanding how the COBRA Toolbox is developed is most important for developers, so beginners may skip this section at first. With an increasing number of contributions from developers around the world, the code base is evolving at a fast pace. The COBRA Toolbox has evolved from monolingual MATLAB software to a multilingual software suite via integration with C, FORTRAN, Julia, Perl and Python code, as well as pre-compiled binaries, for specific purposes. For example, the integration with quadruple precision numerical optimisation solvers, implemented in FORTRAN, for robust and efficient modelling of multi-scale biochemical networks, such as those obtained with integration[60] of metabolic[61] and macromolecular synthesis[62] reconstructions, which represent a new peak in terms of biochemical comprehensiveness and predictive capacity[63]. These developments warranted an industrial approach to software development of the COBRA Toolbox. Therefore, we implemented a continuous integration approach with the aim of guaranteeing a consistent, stable, and high-quality software solution for a broad user community.

The COBRA Toolbox is version controlled using Git (https://git-scm.com), a free and open-source distributed, version control system, which tracks changes in computer files and is used for coordinating work on those files by multiple people. The continuous integration environment facilitates contributions from the fork of COBRA developers to a development branch, whilst ensuring that robust, high-quality, well-tested code is released to end users on the *master* branch. To lower the technological barrier to the use of the aforementioned software development tools, we have developed MATLAB.devTools (https://github.com/opencobra/MATLAB.devTools), a new user-friendly software extension that enables submission of new COBRA software and tutorials. A server-side, semi-automated continuous integration environment ensures that the code in each new submission is first verified automatically, via a comprehensive test suite that detects bugs and integration errors, and second, is reviewed manually by at least one domain expert, before integration with the development branch. Thirdly, each new contribution to the *development* branch is evaluated in practice by active COBRA researchers, before it becomes part of the *master* branch.

Until recently, the code quality checks of the COBRA Toolbox have been primarily *static*: the code has been reviewed by experienced users and developers while occasional code inspections led to discoveries of bugs. The continuous integration setup defined in Figure 2 aims at *dynamic* testing with automated builds, code evaluation, and documentation deployment. Often, a function runs properly independently and yields the desired output(s),

but when called within a different part of the code, logical errors are thrown. The unique advantage of continuous integration is that logical errors are mostly avoided.

Besides automatic testing, manual usability testing is performed regularly by users and is key to provide a tested and usable code base to the end user. These users provide feedback on the usability of the code base, as well as the documentation, and report eventual issues online (https://github.com/opencobra/cobratoolbox/issues). The documentation is automatically deployed to https://opencobra.github.io/cobratoolbox based on function headers. Moreover, each of the narrative tutorials is presented in a format suitable for web browsers (https://opencobra.github.io/cobratoolbox/stable/tutorials).

Controls—COBRA is part of an iterative systems biology cycle[1]. As such, it can be used as a framework for integrative analysis of experimental data in the context of prior information on the biochemical network underlying one or many complementary experimental datasets. Moreover, it can be used to predict the outcome of new experiments, or it can be used in both of these scenarios at once. Assuming all of the computational steps are errorless, the appropriate control for any prediction derived from a computational model is the comparison with independent experimental data, that is, experimental data that was not used for the model-generated predictions. It is also important to introduce quality controls to check that the computational steps are free from certain errors that may arise during adaptation of existing COBRA protocols or development of new ones.

There are various strategies for the implementation of computational quality controls. Within the COBRA Toolbox 3.0, significant effort has been devoted to automatically test the functionality of existing COBRA methods. We have also embedded a large number of *sanity checks,* which evaluate whether the input data could possibly be appropriate for use with a function. These sanity checks have been accumulated over more than a decade of continuous development of the COBRA Toolbox. Their objective is to rule out certain known classes of obviously false predictions that might result from an inappropriate use of a COBRA method, but they do not (and are not intended) to catch every such error, as it is impossible to imagine all of the eventual erroneous inputs that may be presented to a COBRA Toolbox function. It is advisable to add own narratives with additional sanity checks, which will depend heavily on the modelling scenario. Examples of such narratives can be found under https://opencobra.github.io/cobratoolbox/stable/tutorials.

Required expertise

Most of this protocol can be implemented by anyone with a basic familiarity with the principles of constraint-based modelling. Some methods are only for advanced users. If one is a beginner with respect to MATLAB, Supplementary Manual 1 provides pointers to get started. MATLAB is a relatively simple programming language to learn, but it is also a powerful language for an expert due to the large number of software libraries for numerical and symbolic computing that it provides access to. Certain specialised methods within this protocol, such as thermodynamically constraining reaction directionality, depend on the installation of other programming languages and software, which may be too challenging for a beginner with a non-standard operating system.

If the documentation and tutorials provided within the COBRA Toolbox are not sufficient, then Steps 103 and 104 guide the user toward sources of COBRA community support. The computational demands associated with the implementation of this protocol for one's reconstruction or model of choice is dependent on the size of the network concerned. For a genome-scale model of metabolism, usually a desktop computer is sufficient. However, for certain models, such as a community of genome-scale metabolic models, a multi-scale model of metabolism and macromolecular synthesis, or a multi-tissue model, more powerful processing and extensive memory capacity is required, ranging from a workstation to a dedicated computational cluster. Embarrassingly parallel, high-performance computing is feasible for most model analysis methods implemented in the COBRA Toolbox, which will run in isolation with invocation from a distributed computing engine. It is currently an ongoing topic of research, beyond the scope of this protocol, to fully exploit high-performance computing environments with software developed within the wider openCOBRA environment, though some examples[42] are already available for interested researchers to consult.

Limitations

A protocol for the generation of a high-quality, genome-scale reconstruction, using various software applications, including the COBRA Toolbox, has previously been disseminated[6]; therefore, this protocol focuses more on modelling than reconstruction. The COBRA Toolbox is not meant to be a general-purpose computational biology tool as it is focussed on constraint-based reconstruction and analysis. For example, although various forms of generic data analysis methods are available within MATLAB, the input data for integration with reconstructions and models within the COBRA Toolbox is envisaged to have already been preprocessed by other tools. Within its scope, the COBRA Toolbox aims for complete coverage of COBRA methods. The first comprehensive overview of the COBRA methods available for microbial metabolic networks[5] requires an update to encompass many additional methods that have been reported to date, in addition to the COBRA methods targeted toward other biochemical networks. The COBRA Toolbox 3.0 provides the most extensive coverage of published COBRA methods. However, there are certainly some methods that have yet to be incorporated directly as MATLAB implementations, or indirectly via a MATLAB interface to a software dependency. Although in principle any COBRA method could be implemented entirely within MATLAB, it may be more efficient to leverage the core strength of another programming language that could provide intermediate results that can be incorporated into the COBRA Toolbox via various forms of MATLAB interfaces. Such a setup would enable one to overcome any current limitation in coverage of existing methods.

MATERIALS

Equipment setup

Required hardware

- A computer with any 64-bit Intel or AMD processor and at least 8 GB of RAM.

▲ **CRITICAL STEP** Depending on the size of the reconstruction or model, more processing power and more memory may be needed, especially if it is also desired to store the results of analysis procedures within the MATLAB workspace.

- A hard drive with free storage of at least 10 GB.
- **! CAUTION** A working and stable internet connection is required during installation and while contributing to the COBRA Toolbox.

Required software

- A *Linux*, *macOS* or *Windows* operating system that is MATLAB qualified (https://mathworks.com/support/sysreq.html). **! CAUTION** Make sure that the operating system is compatible with the MATLAB version by checking the requirements on https://mathworks.com/support/sysreq/previous_releases.html. Follow the upgrade and installation procedures on the supplier's website or ask your system administrator for help if required.
- MATLAB (MathWorks Inc. - https://mathworks.com/products/matlab.html), version R2014b or above is required. Version R2016a or above is required for running MATLAB live scripts (tutorials *.mlx* files). Note that the tutorials can be run on R2014b using the provided *.m* files. Install MATLAB and its licence by following the official installation instructions (https://mathworks.com/help/install/ug/install-mathworks-software.html) or ask your system administrator. **! CAUTION** No support is provided for versions older than R2014b. MATLAB is released on a twice-yearly schedule. After the latest release (version *b*), it may be a couple of months before certain methods with dependencies on other software become compatible. For example, the latest releases of MATLAB may not be compatible with the existing solver interfaces, necessitating an update of the MATLAB interface provided by the solver developers, or an update of the COBRA Toolbox, or both.
- The COBRA Toolbox (https://github.com/opencobra/cobratoolbox) version 3.0 or above. Install the COBRA Toolbox by following the procedures given on https://github.com/opencobra/cobratoolbox. **! CAUTION** Make sure that all system requirements outlined under https://opencobra.github.io/cobratoolbox/docs/requirements.html are met. If an installation of the COBRA Toolbox is already present, there is no need to re-clone the full repository. Instead, you can update the repository from MATLAB or from the terminal.

  (A) Update from within MATLAB by running:

  ```
  >> updateCobraToolbox
  ```

  (B) Update from the terminal (or shell) by running from within the *cobratoolbox* directory

  ```
  $ cd cobratoolbox # change to the cobratoolbox directory
  $ git checkout master # switch to the master branch
  ```

```
$ git pull origin master # retrieve changes
```

▲ **CRITICAL STEP** The official repository must be cloned as explained in the installation instructions in Steps 97–102. The COBRA Toolbox can only be updated if no changes have been made locally in the cloned repository. Steps 97–102 provide explanations on how to contribute.

In case the update of the COBRA Toolbox fails or cannot be completed, clone the repository again.

- A working *bash* terminal (or shell) with UNIX tools. *curl* version 7.0 or above must be installed to ensure connectivity between the COBRA Toolbox and the remote Github server. The version control software *git* 1.8 or above is required to be installed and accessible through system commands. On *Linux* and *macOS*, a *bash* terminal with *git* and *curl* is readily available. Supplementary Manual 2 provides a brief guide to the basics of using a terminal. **! CAUTION** On *Windows*, the shell integration included with *git Bash* (https://git-for-windows.github.io) utilities must be installed. The command line tools such as *git* or *curl* will be be installed together with *git Bash*. Make sure that you select *<Use git Bash and optional Unix tools from the Windows Command prompt during the installation process>* of git Bash. After installing *git Bash*, restart MATLAB. On *macOS*, a working installation of Xcode (https://developer.apple.com/xcode) version 8.0 or above and command line tools is mandatory. The *Xcode* command line tools may be installed by following the instructions on https://railsapps.github.io/xcode-command-line-tools.html.

Optional software

- Reading and writing models in SBML (Systems Biology Markup Language) format requires the MATLAB interface from the libSBML application programming interface, version 5.15.0 or above. The COBRA Toolbox 3.0 supports the latest SBML Level 3 Flux Balance Constraints Version 2 package (http://sbml.org/Documents/Specifications/SBML_Level_3/Packages/fbc). The libSBML package, version 5.15.0 or above is already packaged with the COBRA Toolbox via the COBRA.binary submodule for all common operating systems. Alternatively, binaries can be downloaded separately and installed by following the procedure on http://sbml.org/Software/libSBML. The COBRA Toolbox developers work closely with the SBML Team to ensure that the COBRA Toolbox supports the latest standards, and moreover that standard development is also focused on meeting the evolving requirements of the constraint-based modelling community. After the latest release of MATLAB, there may be a short time lag before input and output become fully compatible. For example, the input and output of *.xml* files in the SBML standard formats relies on platform dependent binaries that we maintain (https://github.com/opencobra/COBRA.binary) for each major platform, but the responsibility for maintenance of the source code[64] lies with the SBML team (http://sbml.org), who have a

specific forum for raising interoperability issues (https://groups.google.com/forum/#!forum/sbml-interoperability).

- The MATLAB Image Processing Toolbox, the Parallel Computing Toolbox, the Statistics and Machine Learning Toolbox, and the Optimization Toolbox and Bioinformatics Toolbox (https://mathworks.com/products) must be licensed and installed to ensure certain model analysis functionality, such as topology based algorithms, flux variability analysis, or sampling algorithms. The individual MATLAB toolboxes can be installed during the MATLAB installation process. If MATLAB is already installed, the toolboxes can be managed using the built-in MATLAB add-on manager as described on https://mathworks.com/help/matlab/matlab_env/manage-your-add-ons.html.
- The Chemaxon Calculator Plugins (https://chemaxon.com/products/calculator-plugins - Chem-Axon Ltd), version 16.9.5.0 or above, is a suite offering a range of cheminformatics tools. Standardizer is ChemAxon's solution to transform chemical structures into customised, canonical representations to achieve best reliability with chemical databases. The Chemaxon Calculator Plugins, version 16.9.5.0 or above can be installed by following the installation procedures outlined in the user guide on https://chemaxon.com/products/calculator-plugins. A licence is freely available for academics.
- Java (https://java.com/en/download/help/download_options.xml), version 8 or above, is a programming language which enables platform independent applications. Java, version 8 or above, may be installed by following the procedures given on https://java.com/en/download/help/index_installing.xml.
- Python (https://python.org/downloads), version 2.7, is a high-level programming language for general-purpose programming and is required to run NumPy or generate the documentation locally (relevant when contributing). Python, version 2.7 is already installed on *Linux* and *macOS*. On Windows, the instructions on https://wiki.python.org/moin/BeginnersGuide/Download will guide you to install Python.
- NumPy (https://scipy.org/install.html), version 1.11.1 or above, is the fundamental package for scientific computing with Python. NumPy may be installed by following the procedures on https://docs.scipy.org/doc/numpy-1.10.1/user/install.html.
- OpenBabel (https://openbabel.org), version 2.3 or above, is a chemical toolbox designed to speak the many languages of chemical data. OpenBabel may be installed by following the installation instructions on http://openbabel.org/wiki/Category:Installation.
- Reaction Decoder Tool (RDT - https://github.com/asad/ReactionDecoder/releases), version 1.5.0 or above, is a Java-based, open-source atom mapping software tool. The latest version of the Reaction Decoder Tool (RDT) can be installed by following the procedures on https://github.com/asad/ReactionDecoder#installation.

Solvers

- Table 4 provides an overview of supported optimisation solvers. At least one linear programming (LP) solver is required for basic constraint-based modelling methods. Therefore, by default, the COBRA Toolbox installs certain open source solvers, including the LP and MILP solver GLPK (https://gnu.org/software/glpk). However, for more efficient and robust linear optimisation, we recommend to also install an industrial numerical optimisation solver. On *Windows*, the OPTI solver suite (https://inverseproblem.co.nz/OPTI) must be installed separately in order to use the OPTI interface. **! CAUTION** Depending on the type of optimisation problem underlying a COBRA method, an additional numerical optimisation solver may be required.

- Most steps of the solver installation require superuser or administrator rights (sudo) and eventually setting environment variables. Detailed instructions and links to the official installation guidelines for installing Gurobi, Mosek, Tomlab and IBM Cplex can be found on https://opencobra.github.io/cobratoolbox/docs/solvers.html. **! CAUTION** Make sure that environment variables are properly set in order for the solvers to be properly recognised by the COBRA Toolbox.

Application specific software

- Certain solvers have additional software requirements, and some binaries provided in the COBRA.binary (https://github.com/opencobra/COBRA.binary) repository might not be compatible with your system.

- The *dqqMinos* and *Minos* solvers may only be used on Unix. The C-shell *csh* (http://bxr.su/NetBSD/bin/csh) is required. On *Linux* or *macOS*, the C-shell *csh* can be installed by following the instructions on https://en.wikibooks.org/wiki/C_Shell_Scripting/Setup.

- The GNU C-compiler *gcc* 7.0 or above (https://gcc.gnu.org). The library of the gcc compiler is required for generating new binaries of *fastFVA* with a different version of the CPLEX solver than officially supplied. The GNU Fortran compiler *gfortran* 4.1 or above (https://gcc.gnu.org/fortran). The library of the *gfortran* compiler is required for running *dqqMinos.* The *gcc* and *gfortran* compilers can be installed by following the links given on https://opencobra.github.io/cobratoolbox/docs/compilers.html.

Contributing software

- MATLAB.devTools (https://github.com/opencobra/MATLAB.devTools) is highly recommended for contributing code to the COBRA Toolbox in a user-friendly and convenient way, even for those without basic knowledge of *git*. The MATLAB.devTools can be installed by following the instructions given on https://github.com/opencobra/MATLAB.devTools#installation. Alternatively, if the COBRA Toolbox is already installed, then the MATLAB.devTools can be installed directly from within MATLAB by typing:

```
>> installDevTools()
```

PROCEDURE

Initialisation of the COBRA Toolbox ● TIMING 5 – 30 s

1 | At the start of each MATLAB session, the COBRA Toolbox must be initialised. The initialisation can be done either automatically (option A) or manually(option B). For a regular user who primarily uses the official openCOBRA repository, automatic initialisation of the COBRA Toolbox is recommended. It is highly recommended to manually initialise when contributing (see Steps 97–102), especially when the official version and a clone of the fork are present locally.

(A) Automatically initialising the COBRA Toolbox

(i) Edit the MATLAB *startup.m* file and add a line with initCobraToolbox so that the COBRA Toolbox is initialised each time that MATLAB is started.

```
>> edit startup.m
```

(A) Manually initialising the COBRA Toolbox

(i) Navigate to the directory where you installed the COBRA Toolbox and initialise by running:

```
>> initCobraToolbox;
```

▲ **CRITICAL STEP** During initialisation, a check for software dependencies is made and reported to the command window. It is not necessary that all possible dependencies are satisfied before beginning to use the toolbox, e.g., satisfaction of a dependency on a multi-scale linear optimisation solver is not necessary for modelling with a mono-scale metabolic model. However, other software dependencies are essential to be satisfied, e.g., dependency on a linear optimisation solver must be satisfied for any method that uses flux balance analysis. **? TROUBLESHOOTING**

2 | At initialisation, one from a set of available optimisation solvers will be selected as the default solver. If Gurobi is installed, it is used as the default solver for LP, QP, and MILP problems. Otherwise, the GLPK solver is selected for LP and MILP problems. It is important to check if the solvers installed are satisfactory. A table stating the solver compatibility and availability is printed to the user during initialisation. Check the currently selected solvers with

```
>> changeCobraSolver;
```

▲ **CRITICAL STEP** A dependency on at least one linear optimisation solver must be satisfied for flux balance analysis.

Verify and test the COBRA Toolbox ● TIMING ~ 10^3 s

3 | (optional) Test the functionality of the COBRA Toolbox locally. This is recommended if one encounters an error running a function. The test suite runs

tailored tests that verify the output and proper execution of core functions on the locally configured system. The full test suite can be invoked by typing:

```
>> testAll
```

? TROUBLESHOOTING

Importing a reconstruction or a model ● TIMING 10 – 10^2 s

4 | The COBRA Toolbox offers support for several commonly used data formats for describing models, including models in Systems Biology Markup Language (*SBML*), Excel Sheets (*.xls*) and different Simpheny(*c*) formats. The COBRA Toolbox fully supports the standard format documented in the SBML Level 3 Version 1 with the Flux Balance Constraints (*fbc*) package version 2 specifications (www.sbml.org/specifications/sbml-level-3/version-1/fbc/sbml-fbc-version-2-release-1.pdf). In order to load a model with a fileName into the MATLAB workspace as a COBRAv3 model structure, run:

```
>> model = readCbModel(fileName);
```

When filename is left blank, a file selection dialogue window is opened. If no file extension is provided, the code will automatically determine the appropriate format from the given filename. The readCbModel function also supports reading normal MATLAB files for convenience, and checks whether those files contain valid COBRA models. Legacy model structures saved in a *.mat* file are loaded and converted. The fields are also checked for consistency with the current definitions.

▲ **CRITICAL STEP** It is advisable that readCbModel() is used to load new models. This is also valid for models provided in .mat files, as readCbModel checks the model for consistency with the COBRA Toolbox 3.0 field definitions and automatically performs necessary conversions for models with legacy field definitions or field names. In order to develop future-proof code, it is good practice to use readCbModel() instead of the built-in function load. **? TROUBLESHOOTING**

Exporting a reconstruction or a model ● TIMING 10 – 10^2 s

5 | The COBRA Toolbox offers a set of different output methods. The most commonly used formats are SBML *.xml* and MATLAB *.mat* files. SBML is the preferred output format, as it can be read by most applications in the field of computational systems biology. However, some information cannot be encoded in standard SBML, so a *.mat* file might contain information not present in the corresponding SBML output. In order to output a COBRA model structure in either format, use:

```
>> writeCbModel(model, fileName);
```

The extension of the fileName provided is used to identify the type of output requested. The model will consequently be converted and saved in the respective format. When exporting a reconstruction or model, it is necessary that the model adheres to the model structure in

Table 3, and that fields contain valid data. For example, all cells of the the rxnNames field should only contain data of type char and not data of type double. **?**
TROUBLESHOOTING

Use of rBioNet to add reactions to a reconstruction ● TIMING 1 – 10^3 s

6 | We highly recommend using *rBioNet*[48] (a graphical user interface-based reconstruction tool) for the addition or removal of reactions and of gene-reaction associations.

A stoichiometric representation of a reconstructed biochemical network is contained within the model.S matrix. This is a stoichiometric matrix with m rows and n columns. The entry model.S(i,j) corresponds to the stoichiometric coefficient of the i^{th} molecular species in the j^{th} reaction. The coefficient is negative when the molecular species is consumed in the reaction and positive if it is produced in the reaction. If model.S(i,j)== 0, then the molecular species does not participate in the reaction. In order to manipulate an existing reconstruction in the COBRA Toolbox, one can use *rBioNet*, use a spreadsheet, or generate scripts with reconstruction functions. Each approach has its advantages and disadvantages. When adding a new reaction or gene-protein-reaction association *rBioNet* ensures that reconstruction standards are satisfied, but it may make the changes less tractable when many reactions are added. A spreadsheet-based approach is tractable, but only allows for the addition, and not the removal, of reactions. In contrast, using reconstruction functions provides an exact specification for all of the refinements made to a reconstruction. One can also combine these approaches by first formulating the reactions and gene-protein-reaction associations with *rBioNet* and then adding sets of reactions using reconstruction functions.

If you do not have existing *rBioNet* metabolite, reaction, and compartment databases, the first step is to create these files. Please refer to the *rBioNet* tutorial provided in the COBRA Toolbox for instructions on how to add new metabolites and reactions to an *rBioNet* database. Make sure that all the relevant metabolites and reactions that you wish to add to your reconstruction are present in your *rBioNet* databases.

There are two options for using *rBioNet* functionality to add reactions to a reconstruction: using the *rBioNet* graphical interface (option A) or without using the interface (option B). If you wish to add the reactions only to the *rBioNet* database, hence benefiting from the included quality control and assurance measures, but then afterwards use the COBRA Toolbox commands to add reactions to the reconstruction, use option B.

(A) Adding reactions from an *rBioNet* database to a reconstruction using the *rBioNet* graphical user interface.

(i) Verify your *rBioNet* settings

First, make sure the paths to your *rBioNet* reaction, metabolite, and compartment databases are set correctly

```
>> rBioNetSettings;
```

(ii) Load the *.mat* files that hold your reaction, metabolite, and compartment databases.

(iii) To add reactions from an rBioNet database to a reconstruction, invoke the *rBioNet* graphical user interface with:

>> ReconstructionTool;

Select File > Open Model Creator.

(iv) Load your reconstruction by selecting File > Open Model > Complete Reconstruction.

(v) Add reactions from the *rBioNet* database by selecting 'Add Reaction' and selecting a reaction. Repeat for all reactions that should be added to the reconstruction.

(vi) Save your updated reconstruction by selecting File > Save > As Reconstruction Model. As *rBioNet* was created using the old COBRA model structure, use the following command to convert your model to the new model structure:

>> model = convertOldStyleModel(model);

(B) Adding reactions from the *rBioNet* database without using the *rBioNet* interface.

(i) Load (or create) a list of reaction abbreviations ReactionList to be added from the *rBioNet* reaction database:

>> load('Reactions.mat');

(ii) Load the *rBioNet* reaction database 'rxnDB':

>> load('rxnDB.mat');

(iii) Then, add new reactions:

>> for i = 1:length(ReactionList) model = addReaction(model, ReactionList{i}, 'reactionFormula', … rxnDB(find(ismember(rxn(:, 1), ReactionList{i})), 3)); end

Use of a spreadsheet to add reactions to a reconstruction ● TIMING 1 – 10^3 s

7 | Load reactions from a spreadsheet with a pre-specified format[17] into a new model structure modelNewR:

>> modelNewR = xls2model('NewReactions.xlsx');

8 | Merge the existing reconstruction model with the new model structure modelNewR to obtain a reconstruction with expanded content modelNew:

>> modelNew = mergeTwoModels(model, modelNewR, 1);

Use of scripts with reconstruction functions ● TIMING 1 – 10^2s

9 | In order to ensure traceability of all manipulations to a reconstruction, generate, execute and save a script that calls reconstruction functions rather than using the command line. The function addReaction can be used to add a reaction to a reconstruction:

>> model = addReaction(model, ‘GAPDH’, ‘metaboliteList’, {‘g3p[c]’, ‘nad[c]’, ‘pi[c]’, … ‘13bpg[c]’, ‘nadh[c]’, ‘h[c]’}, ‘stoichCoeffList’, [−1; −1; −2; 1; 1; 1]);

The use of metaboliteList provides a cell array of compartment specific molecular species abbreviations, while stoichCoeffList is used to provide a numeric array of stoichiometric coefficients. If particular metabolites do not exist in model.mets, then this function will add them to the list of metabolites. In the function addReaction(), duplicate reactions are recognized even when the order of metabolites or the abbreviation of the reaction are different. Certain types of reactions, such as exchange, sink, and demand reactions[65], may also be added by using the functions addExchangeRxn, addsinkReactions, or addDemandReaction, respectively.

After adding one or multiple reactions to a reconstruction, it is important to verify that these reactions can carry flux; that is, that they are functionally connected to the remainder of the network.

10 | Check whether the added reaction(s) have a nonzero flux value (in other words, can carry flux). To do this for each newly added reaction NewRxn, change it to be the objective function using:

>> model = changeObjective(model, ‘NewRxn’) ;

then maximise ‘max’ and minimise ‘min’ the flux through this reaction.

>> FBA = optimizeCbModel(model, ‘max’) ;

>> FBA = optimizeCbModel(model, ‘min’);

If the reaction should have a negative flux value (e.g., a reversible metabolic reaction or an uptake exchange reaction), then the minimisation should result in a negative objective value FBA.f < 0. If both maximisation and minimisation return an optimal flux value of zero (i.e., FBA.f == 0), then this newly added reaction cannot carry a non-zero flux value under the given simulation condition and the cause for this must be identified.

If the reaction(s) can carry non-zero fluxes, please repeat Steps 20 and 47 to ensure stoichiometric consistency, as well as the chemical and biochemical fidelity.

11 | Remove reactions. In order to remove reactions from a reconstruction, use:

>> modelOut = removeRxns(model, rxnRemoveList);

For example, if manual curation of the literature reveals that a reaction in the generic human metabolic reconstruction, Recon3[66], is not active in a specific cell type being modelled, then one should remove the corresponding reaction from the reconstruction.

12 | Remove metabolites. In order to remove metabolites only, run:

>> model = removeMetabolites(model, metaboliteList, removeRxnFlag);

Note that the removal of one or more metabolites makes sense only if they do not appear in any reactions or if one wishes to remove all reactions associated with one or more

metabolites. For example if a network contains reactions $A + B \Leftrightarrow C$ and $A \Leftrightarrow B$, removing metabolite C will remove the former reaction also.

13 | Remove trivial stoichiometry. If metabolites with zero rows, or reactions with zero columns are present in a stoichiometric matrix, they can be removed with:

```
>> modelOut = removeTrivialStoichiometry(model);
```

After removing one or more reactions (or metabolites) from the reconstruction, please repeat Steps 9 to 13 in order to check that these modifications did not alter existing metabolic functions of the reconstruction-derived models.

Check the scaling of a reconstruction ● TIMING 1 – 10^2 s

14 | Most optimisation solvers are designed to work with data (e.g., stoichiometric coefficients, bounds, and objective coefficients in linear optimisation problems) that is well scaled. Standard solvers are based on 16-digit double-precision floating-point arithmetics, so the input data should not require a solution with more than 8 significant digits in order to ensure that solutions are accurate to the remaining 8 digits of precision. Such a solution approach is sufficient for most metabolic models, except, for instance, if micro and macronutrients are simultaneously being considered. Multi-scale models of metabolism and macromolecular synthesis require higher precision solvers, but they only need to be used when necessary, so it is useful to check the scaling of a new reconstruction or model.

Check the scaling of a stoichiometric matrix with:

```
>> [precisionEstimate, solverRecommendation, scalingProperties] = checkScaling(model);
```

Select a double- or quad-precision optimisation solver ● TIMING 1 – 5 s

15 | The COBRA Toolbox is integrated with a wide variety of different optimisation solvers (cf. Table 4). Quad MINOS[54, 67] is a quadruple-precision version of the general-purpose, industrial-strength linear and nonlinear optimisation solver MINOS. This solver operates with 34 digits of precision, and was developed with multi-scale constraint-based modelling problems in mind. Higher precision solvers are more precise but less computationally efficient than standard solvers. They must be used when necessary, i.e., with multi-scale reconstructions and models. To solve multi-scale linear optimisation problems, the COBRA Toolbox offers a Double-Quad-Quad MINOS method (DQQ) that combines the use of Double and Quad solvers in order to improve efficiency while maintaining high accuracy in the solution. One can set the optimisation solver used by the COBRA Toolbox as solverStatus = changeCobraSolver(solverName, solverType) where solverName specifies the solver to be used, while solverType specifies the type of problems to solve with the solver specified by solverName (‘LP’ for linear optimisation problem, ‘MILP[1] for mixed integer linear problems, ‘QP’ for quadratic problems, ‘MIQP’ for mixed integer quadratic problems, ‘NLP’ for non-linear problems, or ‘ALL’ to change the solver for all

the previously mentioned problem types. Depending on the precisionEstimate, there are two options: choose a double precision solver (option A) or a quad precision solver (option B).

(A) The solverRecommendation is double

(i) If the recommendation shows that a double precision solver is probably sufficient, then, for example, set the Gurobi solver to solve linear programming problems with:

```
>> solverStatus = changeCobraSolver('gurobi', 'LP');
```

A positive solverStatus also indicates that the COBRA Toolbox will use Gurobi as the default linear optimisation solver.

(B) The solverRecommendation is quad

(i) If the recommendation shows that a higher precision solver is required, then, for example, select the quad-precision optimisation solver dqqMinos for solving linear optimisation problems with:

```
>> solverStatus = changeCobraSolver('dqqMinos', 'LP');
```

▲ **CRITICAL STEP** A dependency on at least one linear optimisation solver must be satisfied for flux balance analysis. If any numerical issues arise while using a double precision solver, then a higher precision solver should be tested. For instance, a double precision solver may incorrectly report that an ill-scaled optimisation problem is infeasible although it actually might be feasible for a higher precision solver. The checkScaling function may be used on all operating systems, but the dqqMinos or quadMinos interfaces are only available on UNIX operating systems. **? TROUBLESHOOTING**

Identify stoichiometrically consistent and inconsistent reactions ● TIMING 1 – 10^5s

16 | All biochemical reactions conserve mass; therefore, it is essential that each biochemical reaction in a model does actually conserve mass. Reactions that do not conserve mass[68] are, however, often added to a reconstruction in order to represent the flow of mass into and out of a system, e.g., during flux balance analysis. Every reaction that does not conserve mass, but is added to a model in order to represent the exchange of mass across the boundary of a biochemical system, is henceforth referred to as an *external reaction,* e.g., $D \rightleftharpoons \emptyset$, where $\emptyset$ represents null. Every reaction that is supposed to conserve mass is referred to as an *internal reaction*. Besides exchange reactions, a reconstruction may contain mass imbalanced internal reactions due to incorrect or incompletely specified stoichiometry. This situation results in one or more sets of *stoichiometrically inconsistent* reactions[69]. For instance, the reactions $A + B \rightleftharpoons C$ and $C \rightleftharpoons A$ are stoichiometrically inconsistent because it is impossible to assign a positive molecular mass to all species whilst ensuring that each reaction conserves mass.

By combining flux through both of the former reactions in the forward direction, the net effect is $B \rightarrow \emptyset$, that is, inadvertent exchange of B across the boundary of the model.

In order to distinguish between the reactions in a model that are stoichiometrically consistent and stoichiometrically inconsistent, there are three options: identify reactions with only one stoichiometric coefficient based on the stoichiometric matrix (option A), checking the reactions that are elementally imbalanced based on the chemical formulae of molecular species (option B), or identify the largest set of reactions in a reconstruction that are stoichiometrically consistent (option C).

(A) Use stoichiometric matrix or reaction names

(i) Pinpoint external reactions by identifying reactions with only one stoichiometric coefficient, or reactions with the model.rxns abbreviation prefixes EX_, DM_ and sink_, for exchange, demand and sink reactions, respectively:

```
>> model = findSExRxnInd(model);
```

In the result, model.sIntRxnBool gives a boolean vector of reactions that are heuristically thought to be internal. **? TROUBLESHOOTING**

(B) Use the checkMassChargeBaiance function

(i) When model.metFormulas is populated with the chemical formulae of molecular species, it is possible to check which reactions are elementally imbalanced with:

```
>> [massImbalance] = checkMassChargeBalance(model);
```

The output massImbalance is a $n \times t$ matrix with a non-zero entry for any elemental imbalance in a reaction. The other outputs from this function can also be used to analyse imbalanced reactions to suggest modifications to the stoichiometric specification that can resolve the imbalance. A resolution of mass imbalance should ensure that the reaction stoichiometry is consistent with the known biochemical mechanism of the reaction. **? TROUBLESHOOTING**

(C) Use the findStoichConsistentSubset function

(i) Given stoichiometry alone, a non-convex optimisation problem can be used to approximately identify the largest set of reactions in a reconstruction that are stoichiometrically consistent.

```
>>[~, SConsistentRxnBool, SInConsistentRxnBool,
unknownSConsistencyRxnBool, … model] =
findStoichConsistentSubset(model, massBalanceCheck);
```

When checking for stoichiometric inconsistency, external reactions identified via Option B can be used to warm start the algorithm for Option C if massBalanceCheck == 1. The non-zero entries of unknownSConsistencyRxnBool and unknownSConsistencyMetBool denote reactions and uniquely involved molecular species where consistency could not be established.

▲ **CRITICAL STEP** Any supposedly internal reaction that is actually stoichiometrically inconsistent with the remainder of a reconstruction should be omitted from a model that is intended to be subjected to flux balance analysis, otherwise erroneous predictions may result due to inadvertent violation of the steady-state mass conservation constraint. **? TROUBLESHOOTING**

Identify stoichiometrically consistent and inconsistent molecular species ● TIMING 1 – 10^3 s

17 | Identify the molecular species that only participate in reactions that are stoichiometrically inconsistent using:

```
>>[SConsistentMetBool, ~, SInConsistentMetBool, ~, unknownSConsistencyMetBool, ~, model] = … findStoichConsistentSubset(model, massBalanceCheck);
```

Set simulation constraints ● TIMING 1 – 10^3 s

18 | In order to set the constraints on a model, type

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

The list of reactions for which the bounds should be changed is given by rxnNameList, while the vector vaiue contains the new boundary reaction rate values. This type of bound can be set to a lower (‘l’) or upper bound (‘u’). Alternatively, both bounds can be changed simultaneously (‘b’).

▲ **CRITICAL STEP** The more biochemically realistic the applied constraints are with respect to a particular context, the more likely network states that are specific to that context are to be predicted, as opposed to those predicted from a generic model. All else being equal, a model derived from a comprehensive yet generic reconstruction will be less constrained than a model derived from a less comprehensive yet generic reconstruction. That is, in general, the more comprehensive a reconstruction is, the greater attention must be paid to setting simulation constraints.

Identify molecular species that leak, or siphon, across the boundary of the model ● TIMING 1 – 10^3 s

19 | Identification of internal and external reactions using findSExRxnInd in Step 16A is the fastest option, but may not always be accurate. It is therefore wise to check whether there exist molecular species that can be produced from nothing (leak) or consumed giving nothing (siphon) in a reconstruction, with all external reactions blocked. If modelBoundsFlag == 1, then the leak testing uses the

model bounds on internal reactions, and if modelBoundsFlag == 0, then all internal reactions are assumed reversible.

```
>> modelBoundsFlag = 1;
```

```
>> [leakMetBool, leakRxnBool, siphonMetBool, siphonRxnBool] = ...
findMassLeaksAndSiphons(model, model.SIntMetBool, model.SIntRxnBool,
modelBoundsFlag);
```

▲ **CRITICAL STEP** Non-zero entries in leakMetBool, or siphonMetBool, indicate that the corresponding molecular species can be produced from nothing, or consumed giving nothing, and may invalidate any flux balance analysis prediction.

Identify flux inconsistent reactions ● TIMING 1 – 10^3 s

20 | In flux balance analysis, the objective is to predict reaction fluxes subject to a steady state assumption on internal molecular species and a mass balance assumption for molecular species exchanged across the boundary of the model. It is therefore useful to know, before making any flux balance analysis prediction, which reactions do not admit a non-zero steady state flux, i.e., the reactions that are flux inconsistent, also known as blocked reactions. In order to identify these reactions that do not admit a non-zero flux, use:

```
>>[fluxConsistentMetBool, fluxConsistentRxnBool, fluxInConsistentMetBool, …
fluxInConsistentRxnBool] = findFluxConsistentSubset(model);
```

Flux balance analysis ● TIMING 1 – 10^2 s

21 | In standard notation, flux balance analysis[70] is the linear optimisation problem

$$\begin{aligned} &\max_{v \in \mathbb{R}^n} && \rho(v) := c^T v \quad (1)\\ &\text{s.t.} && S_v = 0,\\ & && l \le v \le u, \end{aligned}$$

where $c \in \mathbb{R}^n$ is a parameter vector that linearly combines one or more reaction fluxes to form the objective function, denoted $\rho(v)$. In the COBRA Toolbox, model.c contains the objective coefficients. $S \in \mathbb{R}^{m \times n}$ is the stoichiometric matrix stored in model.S, and the lower and upper bounds on reaction rates, l, $u \in \mathbb{R}^n$ are stored in model.lb and model.ub, respectively. The equality constraint represents a steady state constraint (production = consumption) on internal metabolites and a mass balance constraint on external metabolites (production + input = consumption + output). The solution to Problem (1) can be obtained using a variety of linear programming (LP) solvers that have been interfaced with the COBRA Toolbox. Table 4 gives the various options. A typical application of flux balance analysis is to predict an optimal steady-state flux

vector that optimises a microbial biomass production rate[71], subject to literature derived bounds on certain reaction rates. Deciphering the most appropriate objective function for a particular context is an important open research question. The objective function in Problem (1) can be modified by changing model.c directly, or using the convenient function:

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

A cell array rxnNameList and numeric array objectiveCoeff are used to give the reaction abbreviation and corresponding linear objective coefficient for one or more reactions to be optimised. By default, objectiveCoeff(p)> 0 and objectiveCoeff(q)< 0 correspond to maximisation and minimisation of the p^{th} and q^{th} reaction abbreviation in rxnNameList.

22 | Flux balance analysis, and many of its variants, can be computed using the versatile function optimizeCbModel. That is, the default method implemented by optimizeCbModel is flux balance analysis, as defined in Problem (1), but depending on the optional arguments provided to optimizeCbModel, many methods that are variations on flux balance analysis are also implemented and accessible with slight changes to the input arguments.

(A) Computing a flux balance analysis solution

(i) A solution to the flux balance analysis Problem (1) can be computed using:

```
>> FBAsolution = optimizeCbModel(model);
```

▲ **CRITICAL STEP** Assuming the constraints are feasible, the optimal objective value FBAsolution.f is unique; however, the optimal flux vector FBAsolution.v is most likely not unique. It is unwise to base any biological interpretation on a single optimal flux vector if it is one of many alternative optima, because the optimal vector returned can vary depending on the solver chosen to solve the problem. Therefore, when a flux vector is interpreted, it should be a unique solution to some optimisation problem.

(B) Computing the unique flux balance analysis solution

(i) In order to predict a unique optimal flux vector, it is necessary to regularise the objective by subtracting a strictly concave function from it. That is $\rho(v) = c^T v - \theta(v)$, where $\theta(v)$ is a strictly convex function. This can be achieved with:

```
>> osenseStr = 'max';
>> minNorm = 1e–6;
>> solution = optimizeCbModel(model, osenseStr, minNorm);
```

Assuming the constraints are feasible, the optimal objective value solution.f and the optimal flux vector solution.v are unique. Setting minNorm to 10^{-6} is equivalent to maximising the functio $\rho(v)$: $= c^T v - \frac{\sigma}{2} v^T v$ with $\sigma = 10^{-6}$ and $\theta(v) = \frac{\sigma}{2} v^T v$ is a regularisation function. With high-dimensional models, it is wise to ensure that the optimal value of the regularisation function is smaller than the optimal value of the original linear objective in Problem (1), that is $\rho\left(v^\star\right) \gg \theta\left(v^\star\right)$. A pragmatic approach is to select minNorm = 1e–6;, then reduce it if necessary.

The solution structure FBAsolution from optimizeCbModel always has the same form, even if the meaning of the fields changes depending on the optional input arguments to the function. The field .stat contains a standardised solver status. If FBAsolution.stat == 1, then an optimal solution has been found and will be returned. The field .v is a flux vector such that the optimal value of the objective function is attained, .y yields the vector of dual variables for the equality constraints, and .w contains the vector of optimal dual variables for the inequality constraints. The field .stat is translated from the solver specific status .origStat. The latter is idiosyncratic to each numerical optimisation solver, and this is translated to the standardised solver status in order to enable other functions within the COBRA Toolbox to operate in a manner invariant with respect to the underlying solver, to the maximum extent possible. If FBAsolution.stat == 2, then the lower and upper bounds are insufficient to limit the value of the objective function and the problem is unbounded, so no optimal solution is returned. If FBAsolution.stat == 0, then the constraints in Problem (1) do not admit any feasible steady state flux vector and therefore no optimal solution exists. If FBAsolution.stat == −1, then no solution is reported, due to a time limit or numerical issues. **? TROUBLESHOOTING**

Relaxed flux balance analysis ● TIMING 1 – 10^3 s

23 | Every solution to Problem (1) must satisfy $S_v = 0$ and $l \leq v \leq u$, independent of any objective chosen to optimise over the set of constraints. It may occur that these constraints are not all simultaneously feasible, i.e., the system of inequalities is infeasible. This situation might be caused by an incorrectly specified reaction bound. In order to resolve the infeasibility, one can use *relaxed flux balance analysis,* which is an optimisation problem that minimises the number of bounds to relax in order to render a flux balance analysis problem feasible. The optimisation problem is

$$\begin{aligned} \min_{v,\,p,\,q} \quad & \alpha\left\|p\right\|_0 + \alpha\left\|q\right\|_0 \\ \text{s.t.} \quad & S_v = 0 \\ & l - p \le v \le u + q \\ & p, q \ge 0, \end{aligned} \tag{2}$$

where p, $q \in \mathbb{R}^n$ denote the relaxations on the lower and upper bounds of the reaction rates vector v, and where $r \in \mathbb{R}^m$ denotes the relaxations of the mass balance constraint. A non-negative vector parameter $\alpha \in \mathbb{R}^n_+$ may be used to prioritise relaxation of bounds on some reactions rather than others, e.g., relaxation of bounds on exchange reactions rather than internal reactions. The optimal choice of parameters depends heavily on the biochemical context. A relaxation of the minimum number of constraints is desirable because, ideally, one should be able to justify the relaxation of each bound with reference to the literature. The scale of this task is proportional to the number of bounds proposed to be relaxed, motivating the sparse optimisation problem to minimise the number of relaxed bounds. Relaxed flux balance analysis can be implemented with:

```
>> solution = relaxFBA(model, relaxOption);
```

The structure relaxOption can be used to prioritise the relaxation of one type of bound over another. For example, in order to disallow relaxation of bounds on all internal reactions, set the field .internalRelax to 0 and to allow the relaxation of bounds on all exchange reactions set the field .exchangeRelax to 2. If there are certain reaction bounds that should not be relaxed, then this can be specified using the boolean vector field .excludedReactions. The first application of relaxFBA to a model may predict bounds to relax that are not supported by literature or other experimental evidence. In this case the field .excludedReactions can be used to disallow the relaxation of bounds on certain reactions.

Sparse flux balance analysis ● TIMING 1 – 10^3 s

24 | The prediction of the minimal number of active reactions required to carry out a particular set of biochemical transformations[72], consistent with an optimal objective derived from flux balance analysis, is based on a cardinality minimisation problem termed *sparse flux balance analysis*

$$\begin{aligned} \min_{v} \quad & \|v\|_0 \\ \text{s.t.} \quad & S_v = b \\ & l \le v \le u \\ & c^T v = \rho^\star, \end{aligned} \tag{3}$$

where the last constraint is optional and represents the requirement to satisfy an optimal objective value $\rho^\star$ derived from any solution to Problem (1). The optimal flux vector can be considered as a steady-state biochemical pathway with minimal support, subject to the bounds on reaction rates and satisfaction of the optimal objective of Problem (1). There are many possible applications of such an approach; here, we consider one example.

Sparse flux balance analysis is used to find the smallest active stoichiometrically balanced cycle that can produce ATP at a maximal rate using the ATP synthase reaction (https://vmh.uni.lu/#reaction/ATPS4m). We use the Recon3Dmodel.mat[66](naming subject to change), which does not have such a cycle active due to bound constraints, but does contain such an active cycle with all internal reactions set to be irreversible. First the model is loaded, then the internal reactions are identified and blocked and finally the objective is set to maximise the ATP synthase reaction rate. Thereafter, the sparse flux balance analysis solution is computed.

```
>> model = readCbModel('Recon3Dmodel.mat');

>> model = findSExRxnInd(model);

>> modelClosed = model;

>> modelClosed.lb(model.SIntRxnBool) = 0;

>> modelClosed.ub(model.SIntRxnBool) = 0;

>> modelClosed_ATPS4mi = changeObjective(modelClosed, 'ATPS4mi', 1);

>> osenseStr = 'max';

>> minNorm = 'zero';

>> sparseFBAsolution = optimizeCbModel(modelClosed_ATPS4mi, osenseStr, minNorm);
```

Identify dead-end metabolites and blocked reactions ● TIMING ~10^2 s

25 | Manually curated as well as automatically created genome-scale metabolic reconstructions contain *dead-end metabolites*, which can either only be produced or only be consumed in the metabolic network (including transport to/from the

system boundary). Given a model, the function detectDeadEnds identifies all *dead-end metabolites* in a model:

```
>> deadEndMetabolites = detectDeadEnds(model);
```

26 | The deadEndMetabolites may be split into downstreamGaps and rootGaps. Metabolites that cannot be produced or consumed by any of the reactions in the network are referred to as rootGaps.

```
>> [deadEndMetabolites, rootGaps, downstreamGaps] = gapFind(model, 'true');
```

Dead-end metabolites listed in deadEndMetabolites are metabolites that are both produced and consumed based on network topology alone but are still dead-end metabolites because there are not any two reactions that can actively produce and consume the metabolite in any steady state.

27 | Both the root and the downstream metabolites are part of reactions that cannot carry any flux (i.e., blocked reactions) given the network topology subject to the current bounds on reaction rates. In order to identify blocked reactions, use:

```
>> blockedReactions = findBlockedReaction(model);
```

Gap fill a metabolic network ● TIMING 10^2 – 10^5 s

28 | Dead-end metabolites show that there are missing reactions in the network that must enable their consumption/production. Thus, they define the boundaries of network gaps that must be filled with one or more reactions to complete our representation of the full metabolic network. These gaps are due to incompleteness of our current knowledge, even in well-studied model organisms[73]. This is partially due to *orphan enzymes*, whose biochemical functions have been described but no corresponding gene sequences have yet been found in any organism[74]. Such biochemical functions cannot be added to reconstructions by automatic (sequence-based) inference, but must be added manually or by some non-sequence related computational approach. Moreover, gene annotations have been experimentally validated in only a limited number of organisms, which may lead to annotation errors when annotations are propagated across a large number of genes using sequence based methods only[75]. Genome-scale metabolic reconstructions can assist in identifying missing knowledge by detecting and filling network gaps, as has been demonstrated for various organisms, including *E. coli*[76, 77], *Chlamydomonas reinhardtii*[78], and *Homo sapiens*[79, 80].

The COBRA Toolbox facilitates the identification and filling of gaps using gapFind[81] and fastGapFill[82]. fastGapFill uses a reference database (***U***, e.g. KEGG REACTION) and a transport and exchange reaction database ***X*** that consists of transport and exchange reactions for each metabolite in both the reference database and the reconstruction. Reactions and pathways are proposed for addition to the metabolic reconstruction during gap filling from the combined ***UX*** database. fastGapFill works for both compartmentalised and decompartmentalised reconstructions. It relies on *fastcc.m*, which was developed within

fastCORE in order to approximate the most compact (i.e., least) number of reactions to be added to fill the highest possible number of gaps.

Prioritise reaction types in the reference database to use for filling gaps using a weights parameter structure. The parameters weights.MetabolicRxns, weights.ExchangeRxns, and weights.TransportRxns allow different priorities to be set for internal metabolic reactions, exchange reactions, and transport reactions, respectively. Transport reactions include intracellular and extracellular transport reactions. The lower the weight for a reaction type, the higher is its priority. Generally, a metabolic reaction should be prioritised in a solution over transport and exchange reactions, with for example:

>> weights.MetabolicRxns = 0.1;

>> weights.ExchangeRxns = 0.5;

>> weights.TransportRxns = 10;

29 | Use the function prepareFastGapFill to prepare a gap filling problem. A reconstruction is given as a model structure along with the optional inputs: list of compartments (listCompartments), a parameter epsilon that is needed for the fastCORE algorithm, the fileName for the universal database (e.g., KEGG; default: *'reaction.lst'*), dictionaryFile, which lists the universal database IDs and their counterpart in the reconstruction as defined in model.mets (default: 'KEGG_dictionary.xls'), and blackList, which permits the exclusion of certain reactions from the universal database (default: no blacklist).

>> [consistModel, consistMatricesSUX, blockedRxns] = …

prepareFastGapFill(model, listCompartments, epsilon, fileName, dictionaryFile, blackList);

The first output variable is consistModel, which contains a flux consistent subnetwork of the input model.

consistMatricesSUX represents the flux consistent **SUX** matrix, which contains the flux consistent S matrix (model), the universal database placed in all cellular compartments along with transport reactions for each metabolite from cytosol to compartment and exchange reactions for all extracellular metabolites. Finally, blockedRxns lists again the blocked reactions in the input model.

30 | The main aim of the fastGapFill function is to find a compact set of reactions from the **UX** matrix to be added to the input model to close the gaps in the model. Gap filling may be carried out using one of two options, depending on the amount of metadata required to aid the interpretation of proposed reactions to be added to the model to fill gaps. The two options are:

(A) Without returning additional metadata

(i) In order to fill gaps without returning additional metadata, run:

>> epsilon = 1e–4;

>> addedRxns = fastGapFill(consistMatricesSUX, epsilon, weights);

The parameter epsilon defines the minimum non-zero flux requested in a blocked reaction when filling gaps. In a multi-scale model, the value of epsilon may need to be decreased, when using a quadruple precision solver (see Step 15). The output addedRxns contains the reactions from the **UX** matrix added to fill the gap(s).

(B) With returning additional metadata

(i) In order to return additional metadata for assistance with the manual evaluation of proposed reactions, use:

>> addedRxnsExtended = postProcessGapFillSolutions(addedRxns, model, blockedRxns);

The output structure addedRxnsExtended contains the information present in addedRxns as well as the statistics and whether desired pathways contain the flux vectors.

The main result is a list of candidate reactions to be added to the metabolic reconstructions. These reactions need to be evaluated for their biological and physiological plausibility in the organism, or cell-type, under consideration.

▲ **CRITICAL STEP** Algorithmic approaches help to identify new candidate reactions, but these candidates must be manually curated before being added to a reconstruction. This step is critical for obtaining a high-quality metabolic reconstruction. Adding the least number of reactions to fill gaps may not be the most appropriate assumption from a biological viewpoint. Consequently, the reactions proposed to be added to reconstruction require further manual assessment. Proposed gap filling solutions must be rejected if they are biologically incorrect.

The mapping between the metabolite abbreviations in the universal database (e.g., KEGG) and the reconstruction metabolite abbreviations in model.mets, will ultimately limit how many blocked reactions might be resolved with fastGapFill. The larger the number of metabolites that map between these different namespaces, the larger the pool of metabolic reactions from the universal database that can be proposed to fill gaps. The mapping between the reconstruction and universal metabolite database can be customised using the dictionaryFile, which lists the universal database identifiers and their counterparts in the reconstruction.

Extracellular metabolomic data ● TIMING 10^3 – 10^5 s

31 | *Metabolomics* is an indispensable analytical method in many biological disciplines including microbiology, plant sciences, biotechnology, and biomedicine. In particular, extracellular metabolomic data are often generated from cell lines in order to characterise and phenotype them under different experimental conditions (e.g., drug treatment or hypoxia). However, the analysis and interpretation of metabolomic data is still in its infancy, limiting the interpretation to potential metabolic pathways rather than providing a comprehensive understanding of the underlying mechanistic basis of the observed data.

MetaboTools is a COBRA Toolbox extension[65], that integrates semi-quantitative and quantitative extracellular metabolomic data with metabolic models. The resulting models allow for the interpretation and generation of experimentally testable mechanistic hypotheses. With *MetaboTools*, extracellular metabolomic data are integrated with a COBRA model structure, e.g., the generic human metabolic model[66], in a way that ensures the integration of a maximal number of measured metabolites, while adding a minimal number of additional uptake and secretion metabolites such that the specified constraints on the metabolic network can be sustained.

It is assumed that the extracellular metabolomic experiments are carried out with a defined fresh medium and that the corresponding model can only take up the components of the medium (plus dissolved gases). To apply constraints that are representative of the chemical composition of the fresh medium used in an experiment, use the setMediumConstraints function:

>> modelMedium = setMediumConstraints(starting_model, set_inf, current_inf, … medium_composition, met_Conc_mM, cellConc, t, cellWeight, mediumCompounds, … mediumCompounds_lb);

The starting_model is the model before addition of fresh medium constraints. The current_inf input argument allows one to specify a value for the large magnitude finite number that is currently used to represent an effectively infinite reaction rate bound, then harmonise them to a new value specified by set_inf. When no information on the bounds of a reaction is known, the ideal way to set reaction bounds is model.lb(j)= −inf; and model.ub(j)= inf;. However, depending on the optimisation solver, an infinite lower or upper bound may or may not be accepted. Therefore, when no information on the bounds of a reaction are known, except perhaps the directionality of the reaction, then the upper or lower bound may be a large magnitude finite number, e.g., model.ub(j)= 1000;.

The fresh medium composition must be specified with a vector of exchange reaction abbreviations for metabolites in the cell medium medium_composition and the corresponding millimolar concentration of each medium component met_Conc_mM. The density of the culture (cellConc, cells per mL), the time between the beginning and the end of the experiment (t, hours), and the measured cellular dry weight (cellWeight, gDW) must also be specified. Basic medium components (mediumCompound), such as protons, water

and bicarbonate, and the corresponding lower bounds on exchange reactions (mediumCompounds_lb), must also be specified. Even though they are present, they are not usually listed in the specification of a commercially defined medium, but they are needed for cells and the generic human metabolic model in order to support the synthesis of biomass. The modelMedium is a new model with external reaction bounds set according to the defined fresh medium.

32 | Next, prepare the quantitative exometabolomic data using the prepIntegrationQuant function:

```
>> prepIntegrationQuant(modelMedium, metData, exchanges, sampleNames, test_max, test_min, … outputPath);
```

The fluxes for each metabolite are given as uptake (negative) and secretion (positive) flux values in a metabolomic data matrix metData, in which each column represents a sample in sampleNames and each row in exchanges represents an exchanged metabolite. The units used for fluxes must be consistent within a model. For the input model in modelMedium, the prepintegrationQuant function tests whether the qualitative uptake (test_max, e.g., +/−500) and secretion (test_min, e.g., 10^{-5}) of the metabolites is possible for each sample defined in the metabolomic data matrix metData. If a metabolite cannot be secreted or taken up, it will be removed from the data matrix for that particular sample. Possible reasons for this could be missing production or degradation pathways, or blocked reactions. For each sample, the uptake and secretion profile compatible with the input model in modelMedium is saved to the location specified in outputPath using the unique sample name.

33 | The model constrained by the defined fresh medium composition modelMedium and the output of the prepintegrationQuant function can now be used to generate a set of functional, contextualised, condition-specific models using:

```
>> [ResultsAllCellLines, OverViewResults] = setQuantConstraints(modelMedium, samples, tol, … minGrowth, obj, no_secretion, no_uptake, {}, {}, 0, outputPath);
```

A subset of samples can be specified with samples. All fluxes smaller than tol will be treated as zero. A lower bound (minGrowth, e.g., 0.008 per hour) on a specified objective function, e.g., obj = biomass_reaction2; needs to be defined, along with metabolites that should not be secreted, e.g., no_secretion = ‘EX_o2[e]’, or taken up (no_uptake = ‘EX_o2s’). The function returns a ResultsAllCellLines structure containing the context-specific models as well as an overview of model statistics in OverViewResults. For each sample, a condition-specific model is created, in which the constraints have been set in accordance with the medium specification and the measured extracellular metabolomic data. This set of condition-specific models can then be phenotypically analysed using the various additional functions present in the COBRA Toolbox as detailed in the *MetaboTools* protocol[65].

Intracellular metabolomic data ● TIMING 10^2 – 10^4 s

34 | COBRA methods have also been developed for integration with intracellular metabolomic measurements[83–85], further improving the ability of the COBRA Toolbox to be used for the integration and interpretation of metabolomic data. In particular, unsteady-state flux balance analysis (uFBA[85]) enables the integration

of absolutely quantified time-course metabolomic data into a metabolic model, generating constraints on intracellular fluxes even when intracellular metabolite levels are not at steady-state. The main steps in the ufba method are illustrated in Figure 3.

The first step is to experimentally quantify the absolute concentrations of a set of extracellular and intracellular metabolites at regular time intervals[85].

35 | Plot the time-course metabolomic data. If the data is non-linear, use principal component analysis to define a sequence of temporal stages during which the time-course metabolomic data can be considered piecewise linear.

36 | Use linear regression to estimate the rate of change of concentration with respect to time for each measured metabolite and for each temporal stage.

37 | Load a standard COBRA model structure containing the fields .s, .b, .lb, .ub, .mets, and .rxns.

38 | Integrate the rate of change in concentration for each measured metabolite with a COBRA model with:

```
>> uFBAoutput = buildUFBAmodel(model, uFBAvariables);
```

The uFBAvariables structure must contain the following fields: .metNames is a list of measured metabolites, .changeslopes provides the rate of change of concentration with respect to time for each measured metabolite, .changeintervals yields the difference between the mean rate of change of concentration with respect to time and the lower bound of 95% percent confidence interval. The list ignoreSlopes contains metabolites whose measurements should be ignored due to insignificant rate of change.

The output is a uFBAoutput structure that contains the following fields: .model, a COBRA model structure with constraints on the rate of change of metabolite concentrations, .metsToUse with a list of metabolites with metabolomic data integrated into the model, and .relaxedNodes with a list of metabolites that deviate from steady-state along with the direction (i.e., accumulation or depletion) and magnitude (i.e., reaction bound) of deviation. The uFBA algorithm automatically determines sink or demand reactions needed to return a model with at least one feasible flux balance solution, by automatically reconciling potentially incomplete or inaccurate metabolomic data with the model structure. The added sink or demand reactions allow the corresponding metabolites, defined by .relaxedNodes, to deviate from a steady state to ensure model feasibility. The default approach is to minimise the number of metabolites that deviate from steady state.

The buildUFBAmodel function integrates quantitative time course metabolomic data with a model by setting rates of change with respect to time for a set of measured intracellular and extracellular metabolites. A set of sink reactions, demand reactions, or both, may have been added to certain nodes in the network to ensure that the model admits at least one feasible mass balanced flux.

39 | The obtained model can then be minimized using optimizeCbModel:

```
>> model_ufba = optimizeCbModel(uFBAoutput.model);
```

Integration of transcriptomic and proteomic data ● TIMING 10^2 – 10^4 s

40 | Given a generic reconstruction of a biochemical network for a particular organism, some reactions may only be active in a specific tissue, cell-type, or under specific environmental conditions. It is necessary to extract a context-specific model from a generic model in order to create a model that is representative of the part of the biochemical network that is active within a particular context. Each context-specific model is therefore a subset of a generic model. A variety of experimental data can be used to determine the set of reactions that must be part of a context-specific model, including transcriptomic, proteomic, and metabolomic data, as well as complementary experimental data from the literature.

Several model extraction methods have been developed, with different underlying assumptions, and each has been the subject of multiple comparative evaluations[86–88]. The selection of a model extraction method and its parametrisation, as well as the methods chosen to preprocess and integrate the aforementioned omics data, significantly influences the size, functionality, and accuracy of the resulting context-specific model. Currently, there is insufficient evidence to assert that one model extraction method universally gives the most physiologically accurate models. Therefore, a pragmatic approach is to test the biochemical fidelity of context-specific models generated using a variety of model extraction methods.

The COBRA Toolbox offers six different model extraction methods, accessible via a common interface:

```
>> tissueModel = createTissueSpecificModel(model, options);
```

The different methods and associated parameters are selected via the options structure. The .solver field indicates which method shall be used. The other fields of the options structure vary depending on the method and often depend on bioinformatic preprocessing of input omics data. There are additional optional parameters for all algorithms, with the default being the values indicated in the respective papers. Please refer to the original papers reporting each algorithm for details on the requirements for preprocessing of input data. Each of the six different model extraction methods can be invoked using:

(A) The FASTCORE[89] algorithm

- **(i)** One set of core reactions that is guaranteed to be active in the extracted model is identified by FASTCORE. Then, the algorithm finds the minimum number of reactions possible to support the core; .core field provides the core reactions which have to be able to carry flux in the resulting model.

(B) The GIMME[90] algorithm

- **(i)** With this algorithm, the usage of low-expression reactions is minimised while keeping the objective (e.g., biomass) above a certain value; .expressionRxns field provides the reaction expression, with −1

for unknown reactions or reactions not linked to genes; .threshold field sets the threshold above which a reaction is assumed to be active.

(C) The iMAT[91] algorithm

(i) iMAT finds the optimal trade-off between including high-expression reactions and removing low-expression reactions; .expressionRxns field is defined as above; .threshold_lb field is the threshold below which reactions are assumed to be inactive; .threshold_ub field is the threshold above which reactions are assumed to be active.

(D) The INIT[92] algorithm

(i) The optimal trade-off between including and removing reactions based on their given weights is determined by this algorithm; .weights field provides the weights w for each reaction in the objective of INIT $\left(max\left(\sum_{i \in R} w_i y_i + \sum_{j \in M} x_j\right)\right)$. Commonly a high expression leads to higher positive values and low or no detection leads to negative values.

(E) The MBA[93] algorithm

(i) MBA defines high-confidence reactions to ensure activity in the extracted model. Medium confidence reactions are only kept when a certain parsimony trade-off is met. .medium_set field provides the set of reactions that have a medium incidence, while .high_set field provides the set of reactions that have to be in the final model. Any reaction not in the medium or high set is assumed to be inactive and preferably not present in the final model.

(F) The mCADRE[94] algorithm

(i) A set of core reactions is first found and all other reactions are then pruned based on their expression, connectivity to core, and confidence score. Reactions that are not necessary to support the core or defined functionalities are thus removed. Core reactions are removed if they are supported by a certain number of zero-expression reactions. .confidenceScores field provides reliability for each reaction, generally based on literature, while .ubiquityScore field provides the ubiquity score of each reaction in multiple replicates, i.e., the number of times the reaction was detected as active in experimental data under the investigated condition.

▲ **CRITICAL STEP** When integrating omics data, parameter selection is critical, especially the threshold for binary classification, e.g., the threshold for genes into active or inactive sets. Algorithmic performance often strongly depends on parameter choices and on the choice of data preprocessing method[87]. However, createTissueSpecificModel does not offer data preprocessing tools, because the selection of the discretisation method and parameters depend on the origin of the data. However, the COBRA Toolbox offers

functionality to map preprocessed expression data to reactions via the function mapExpressionToReactions(model, expression).

Adding biological constraints to a flux balance model ● TIMING ~ 10^2 s

41 | A cell-type or organ-specific model can be converted into a condition-specific model, based on the imposition of experimentally derived constraints. There are several types of constraints that can be imposed on a metabolic network, such as biomass maintenance requirements, environmental constraints, or maximal enzyme activities. In general, biomass constraints[71] are added as part of a biomass reaction. In some instances, however, a cell-type (e.g., neuron) does not divide, but is only required to turn over its biomass components. Turnover rates are commonly expressed as half-lives and represent the time required for half of the biomass precursor to be replaced[95]. A model can be constrained with inequality constraints so as to require a minimal rate of turnover for a metabolite. If that metabolite possesses only one degradation pathway, then it is sufficient to adjust the bounds on a reaction in that pathway. However, if there are multiple possible degradation pathways, then it is necessary to impose a lower bound on the total rates of a set of irreversible degradation reactions, one for each possible degradation pathway of the metabolite in question.

The implementation of such a constraint is illustrated in the following example. In the brain, phosphatidylcholine (PC) can be degraded by three different metabolic pathways[96]:

- Phospholipase D acts on the choline/phosphate bond of PC to form choline and phosphatidic acid (PCHOLP_hs, https://vmh.uni.lu/#reaction/PCHOLP_hs).
- Phospholipase A2 acts on the bond between the fatty acid and the hydroxyl group of PC to form a
- fatty acid (e.g., arachidonic acid or docosahexaenoic acid) and lysophosphatidylcholine (PLA2_2, https://vmh.uni.lu/#reaction/PLA2_2).
- Ceramide and PC can also be converted to sphingomyelin by sphingomyelin synthetase (SMS, https://vmh.uni.lu/#reaction/SMS).

Load a COBRA model and define the set of reactions that will represent degradation of the metabolite in question:

```
>> multipleRxnList = {'PCHOLP_hs', 'PLA2_2', 'SMS'};
```

▲ **CRITICAL STEP** Correctly converting the literature data into bound constraints with the same units used for the model fluxes may be a challenge. Indeed, the curation of biochemical literature to abstract the information required to quantitatively bound turnover rates can take between 4–8 weeks, when the target is to retrieve the biomass composition and the turnover rates of each of the different biomass precursors. Once all the constraints are available, imposing the corresponding reaction bounds takes less than 5 minutes.

42 | Verify that all the reactions are irreversible (the lower and upper bounds should be greater or equal to 0).

```
>> rxnInd = findRxnIDs(model, multipleRxnList);

>> model.lb(rxnInd);

>> model.ub(rxnInd);
```

43 | Generate and add the constraint:

```
>> c = [1, 1, 1];

>> d = 2.674;

>> ineqSense = 'G';

>> modelConstrained = constrainRxnListAboveBound(model, multipleRxnList, c, d,
ineqSense);
```

where c is a vector forming the inequality constraint $c^T v \geq d$, and d is a scalar. ineqSense encodes the sense of these inequality ('L' for a lower inequality, or 'G' for an upper inequality). In this example, all entries of c are positive as we seek for the sum of the rates of the three reactions (irreversible in the forward direction) to be greater than d. This extra constraint is encoded in the model.C field.

44 | Check that the constraints are correctly added to the model:

```
>> [nMet, nRxn] = size(modelConstrained.S);
```

45 | Solve the FBA problem with the extra constraint $c^T v \geq d$:

```
>> solution = optimizeCbModel(modelConstrained, 'max', 1e–6);
```

46 | Check the values of the added fluxes. The sum of fluxes should be greater than or equal to the value of d:

```
>> solution.v(rxnInd);

>> sum(c*FBAsolution.v(rxnInd));
```

Qualitative chemical and biochemical fidelity testing ● TIMING 10^2 – 10^3 s

47 | Once a context-specific model is generated, it is highly advisable to frequently compare preliminary model predictions with published experimental data[6]. Such predictions must be compared directly with an unbiased selection of appropriate independent biological literature in order to identify possible sources of misconception or computational misspecification. It is challenging to compare genome-scale predictions with experimental data that may only be available for a subset of a biochemical network. In this context, it is important to first turn to literature relevant to the aspect of the biological network being represented by a model and then check if the literature result is correctly predicted by the model. Inevitably, this is an iterative approach with multiple rounds of iterative refinement of the reconstruction and the model derived from it, before finalising

a model version and comparison of final predictions with independent experimental data.

A draft model should be subjected to a range of quantitative and qualitative chemical and biochemical fidelity tests. As described in Step 16, chemical fidelity testing includes testing for stoichiometric consistency. This should not be necessary if one starts with a stoichiometrically consistent generic model and extracts a context-specific model from it. However, it is possible that misspecified reactions might have been inadvertently added during refinement of a reconstruction, therefore retest for stoichiometric consistency. Beyond chemical fidelity, it is advised to test again for biochemical fidelity. Such tests are very specific to the particular biological domain that is being modelled. Here we focus on human metabolism and use modelClosed, the Recon3Dmodel[66] with all external reactions closed, from Steps 21–22.

It is important to encode and conduct qualitative fidelity tests for anticipated true negatives. The following test is for the production of ATP from water alone in a closed model:

```
>> modelClosedATP = changeObjective(modelClosed, 'DM_atp[c]');

>> modelClosedATP = changeRxnBounds(modelClosedATP, 'DM[atp_c]', 0, 'l');

>> modelClosedATP = changeRxnBounds(modelClosedATP, 'EX_h2o[e]', −1, 'l');

>> FBAsol = optimizeCbModel(modelClosedATP);
```

If FBAsol.stat == 0, then the model is incapable of producing ATP from water, as expected. If FBAsol.stat == 1, then the supposedly closed model can produce ATP from water. This indicates that there are stoichiometrically inconsistent reactions in the network, which need to be identified. See Step 16 for instructions how to approach this analysis.

48 | It is also important to encode and conduct qualitative fidelity tests for anticipated true positives. The following metabolic function test is for the production of mitochondrial succinate from 4-Aminobutanoate in a model that is closed to exchange of mass across the boundary of the system, except for the metabolites 'gly[c]', 'co2[c]',and 'nh4[c]'.

```
>> modelClosed = addSinkReactions(modelClosed, {'gly[c]', 'co2[c]', 'nh4[c]'}, …

[−100, −1; 0.1, 100; 0.1, 100]);

>> modelClosed = changeObjective(modelClosed, 'sink_nh4[c]');

>> sol = optimizeCbModel(modelClosed, 'max', 'zero');
```

If FBAsol.stat == 1, then it is feasible for the model to produce mitochondrial succinate from 4-Aminobutanoate. If FBAsol.stat == 0, then this metabolic function is infeasible. This is not anticipated and indicates that further gap filling is required (see Steps 28–30).

Quantitative biochemical fidelity testing ● TIMING 10^2 – 10^3 s

49 | It is important to check if a model can reproduce or closely approximate known quantitative features of the biochemical network being represented. Here we illustrate how to predict the ATP yield from different carbon sources under aerobic or anaerobic conditions for Recon3D[66]. These are compared with the values for the corresponding ATP yields obtained from the biochemical literature. This approach can be adapted for condition- and cell-type specific models derived from Recon3D in order to test whether these models are still able to produce physiologically relevant ATP yields. Add and define the ATP hydrolysis reaction DM_atp[c] to be the objective reaction in the model with:

```
>> modelClosed = addReaction(modelClosed, ...

'DM_atp[c]', 'h2o[c] + atp[c] -> adp[c] + h[c] + pi[c]');

>> modelClosed = changeObjective(modelClosed, 'DM_atp[c]');
```

50 | Allow the model to uptake oxygen and water, then provide 1 mol/gdw/hr of a carbon source, e.g., glucose (VMH ID: glc_D[e]):

```
>> modelClosed.lb(find(ismember(modelClosed.rxns, 'EX_o2[e]'))) = −1000;

>> modelClosed.lb(find(ismember(modelClosed.rxns, 'EX_h2o[e]'))) = −1000;

>> modelClosed.ub(find(ismember(modelClosed.rxns, 'EX_h2o[e]'))) = 1000;

>> modelClosed.ub(find(ismember(modelClosed.rxns, 'EX_co2[e]'))) = 1000;

>> modelClosed.lb(find(ismember(modelClosed.rxns, 'EX_glc_D[e]'))) = −1;

>> modelClosed.ub(find(ismember(modelClosed.rxns, 'EX_glc_D[e]'))) = −1;
```

51 | Compute a flux balance analysis solution with maximum flux through the DM_atp[c] reaction:

```
>> FBAsolution = optimizeCbModel(modelClosed, 'max', 'zero');
```

MinSpan Pathways: a sparse basis of the nullspace of a stoichiometric matrix ● TIMING 10^2 – 10^4 s

52 | COBRA models are often mathematically deconstructed into feasible steady state flux vectors in biochemical networks that can be biologically conceptualized as pathways. Much development and analysis has been done for such *pathway vectors* in terms of elementary flux modes[97], extreme pathways[98], and elementary flux vectors[99]. As the number of elementary or extreme vectors scales exponentially with the size of a typical metabolic network, increasingly efficient algorithms become essential for enumerating elementary or extreme vectors at genome-scale. An alternate approach[100] is to approximately compute a set of n – rank(S) sparse linearly independent flux vectors that together form a basis of the right nullspace of a stoichiometric matrix and also satisfy specified

constraints on reaction directionality. This approach requires the solution of a greedy sequence of mixed-integer linear optimisation problems, each of which computes a sparse flux mode that is linearly independent from the rest of the vectors within a nullspace basis. The end result is a sparse set of linearly independent flux modes denoted *MinSpan* pathways.

Given a model with a stoichiometric matrix, reaction bounds and reaction identifiers, the *MinSpan* algorithm may be invoked with:

```
>> Z = detMinSpan(model, params);
```

The params structure provides the user ability to change key parameters. Among others, the main parameters include the amount of time .timeLimit for each iterative solve in seconds and the number of threads for the MILP solver to use. The output $Z \in \mathrm{R}^{n \times (n\text{-rank}(S))}$ is a sparse set of n – rank(S) linearly independent flux modes, each corresponding to a *MinSpan* pathway.

Low dimensional flux variability analysis ● TIMING 1 – 10^3 s

53 | Flux balance analysis does not, in general, return a unique optimal flux vector. That is, Problem (1) returns an optimal flux vector, $v^\star \in \mathbb{R}^n$ with one flux value for each reaction, but typically an infinite set of steady state flux vectors exist that can satisfy the same requirement for an optimal objective, $c^T v^\star = c^T v$, as well as the other equalities and inequalities in Problem (1). Flux variability analysis is a widely used method for evaluating the minimum and maximum range of each reaction flux that can still satisfy the aforementioned constraints using two optimisation problems for each reaction of interest

$$\begin{aligned} \max_{v} \backslash \min \quad & v_j \qquad (4) \\ \text{s.t.} \quad & S_v = 0, \\ & l \le v \le u, \\ & c^T v = c^T v^\star . \end{aligned}$$

Just as there are many possible variations on flux balance analysis, there are many possible variations on flux variability analysis. The COBRA Toolbox offers a straightforward interface to implement standard flux variability analysis and a wide variety of options to implement variations on flux balance analysis.

(A) Standard flux variability analysis

(i) The following command can be invoked to compute standard flux variability analysis:

```
>> [minFlux, maxFlux] = fluxVariability(model);
```

The result is a pair of n dimensional column vectors, minFlux and maxFlux, with the minimum and maximum flux values satisfying Problem (4).

(B) Advanced flux variability analysis

(i) The full spectrum of flux variability analysis options can be accessed using the command:

>> [minFlux, maxFlux, Vmin, Vmax] = fluxVariability(model, optPercentage, osenseStr, … rxnNameList, verbFlag, allowLoops, method);

The optPercentage parameter allows one to choose whether to consider solutions that give at least a certain percentage of the optimal solution. For instance optPercentage = 0 would just find the flux range of each reaction, without of any requirement to satisfy any optimality with respect to flux balance analysis. Setting the parameters osenseStr = 'min' or osenseStr = 'max' determines whether the flux balance analysis problem is first solved as a minimisation or maximisation. The rxnNameList accepts a cell array list of reactions to selectively perform flux variability upon. This is useful for high dimensional models, for which the computation of a flux variability for all reactions is more time consuming. The additional $n \times k$ output matrices Vmin and Vmax return the flux vector for each of the $k \leq n$ fluxes selected for flux variability. The verbFlag input determines how much output shall be printed. The parameter allowLoops == 0 invokes a mixed integer linear programming implementation of thermodynamically constrained flux variability analysis for each minimisation or maximisation of a reaction rate. The method input argument determines whether the output flux vectors also minimise the 0–norm, 1–norm or 2–norm while maximising or minimising the flux through one reaction.

The default result is a pair of maximum and minimum flux values for every reaction. Optional parameters may be set. For instance, parameters can be set to control which subset of $k \leq n$ reactions of interest that shall be be obtained, or to determine the characteristics of each of the $2 \times k$ flux vectors.

High dimensional flux variability analysis ● TIMING 1 – 10^5 s

54 | Besides flux balance analysis, flux variability analysis is the most widely used constraint-based modelling method for high-dimensional models. However, its use in this setting requires a more sophisticated computational approach, with a multi-core processor[101], or computational cluster[42], and a commercial-grade linear optimisation solver. In this setting, advanced users have two options:

(A) Use fastFVA with MATLAB

(i) Solve the $2 \times k$ linear optimisation problems using multiple threads running on parallel processors with fastFVA, which depends on the CPLEX solver (IBM Inc.), using the command :

```
>> [minFlux, maxFlux, optsol] = fastFVA(model,
optPercentage, osenseStr);
```

The output argument optsol returns the optimal solution of the initial FBA.

(B) Use *distributedFBA.jl* with Julia

(i) An alternative is to solve the $2 \times k$ linear optimisation problems using multiple threads running on parallel processors or a cluster using *distributedFBA.jl*, an openCOBRA extension that permits the solution of flux balance analysis, a distributed set of flux balance problems, or a flux variability analysis using a common of solver (GLPK, CPLEX, Clp, Gurobi, Mosek). Assuming that *distributedFBA.jl* has been correctly installed and configured, the commands to go back and forth between a model or results in MATLAB and the computations in Julia are:

```
>> save('high_dimensional_model.mat', unimodel);

# -- switch to Julia --

julia> model =
loadModel("high_dimensional_model.mat");

julia> workersPool, nWorkers = createPool(128);

julia> minFlux, maxFlux, optSol, fbaSol, fvamin,
fvamax = distributedFBA(model, … solver, nWorkers =
nWorkers, optPercentage = optPercentage, preFBA =
true); julia>
saveDistributedFBA("high_dimensional_FVA_results.m
at");

# -- switch to MATLAB --

>> load('high_dimensional_FVA_results.mat');
```

Here, nWorkers = 128 will distribute the flux variability analysis problem amongst 128 Julia processes on one or more computing nodes in a computational cluster.

Uniform sampling of steady-state fluxes ● TIMING 1 – 10^3 s

55 | An unbiased characterisation of the set of flux vectors consistent with steady state, mass balance, and reaction bound constraints can be obtained by uniformly sampling the *feasible set* $\Omega := \{v | Sv = 0; l \leq v \leq u\}$. The feasible set for sampling should be defined based on biochemically justifiable constraints. These are the same conditions that apply when formulating the flux balance analysis Problem (1), except that there is no need to formulate a linear objective.

To ensure the sample is statistically representative of the entire feasible set, a sufficiently large number of flux vectors and the flux vectors must be collected randomly within the feasible set. Recently, we distributed new software to uniformly sample feasible sets of steady state fluxes[102] based on a *coordinate hit-and-run with rounding* (CHRR) algorithm[103, 104] that is guaranteed to return a statistically uniform distribution when appropriately utilised. The CHRR sampling algorithm is therefore used by default. Figure 4 illustrates the basics of this algorithm.

Sampling of a model is invoked either by using the default setting or by tailoring the parameters with more arguments to the interface. A pragmatic approach is to first try Option (A) with the default parameters, then check the quality of the marginal flux distribution for a subset of reactions (see Figure 4). Especially for higher dimensional models, it may be necessary to tune the parameters with Option (B).

(A) Sampling of a mono-scale model with $\lesssim$ 2000 variables

(i) A Model that contains less than 2000 variables can usually be sampled using the default settings:

```
>> [modelSampling, samples] = sampleCbModel(model);
```

The samples output is an $n \times p$ matrix of sampled flux vectors, where p is the number of samples. In order to accelerate any future rounds of sampling, use the modelSampling output. This is a model storing extra variables acquired from preprocessing the model for sampling (see Figure 4).

(B) Sampling of a model with $\lesssim$ 10000 variables

(i) Larger Models containing less than 10000 variables may be sampled by tuning the optional input parameters:

```
>> [modelSampling, samples] = sampleCbModel(model,
sampleFile, samplerName, options, … modelSampling);
```

The variable sampleFile contains the name of a *.mat* file used to save the sample vectors to disk. A string passed to samplerName can be used to sample with non-default solvers. The options structure contains fields that control the sampling density (.nSkip) and the number of samples (.nSamples). The total number of samples returnedis $p = nSkip \times nSamples$. The output modelSampling may be used in subsequent rounds of sampling. Although rounding large models is computationally demanding, the results can be reused when sampling the same model more than once. The CHRR algorithm provably converges to a uniform stationary sampling distribution if enough samples are obtained and has been tested with mono-scale metabolic models with up to 10000 reactions. The default parameters are set using heuristic rules to estimate a sufficiently large number of samples, which

balances this requirement against the desire to complete the sampling procedure in a practically useful period of time. **?** **TROUBLESHOOTING**

Identify all genetic manipulations leading to targeted overproductions ● TIMING 10 –10^5 s

56 | A variety of strain design algorithms[58] are implemented within the COBRA Toolbox, including OptKnock[105], OptGene[106], Genetic Design Local Search (GDLS[107]), and OptForce[108]. While OptKnock, OptGene, and GDLS could identify gene deletion strategies, the OptForce method can identify not only gene deletion but also up- and down-regulation strategies. As the OptForce method is new to this version of the COBRA Toolbox, we provide an illustrative example of strain design using this method.

Consider the problem of finding a set of interventions of size κ such that when these interventions are applied to a wild-type strain, the mutant created will produce a particular target of interest at a higher yield than the wild-type strain. The interventions could be knock-outs (zero out the flux for a particular reaction), up-regulations (increase the flux for a particular reaction), or down-regulations (decrease the flux for a particular reaction). As an example, we will use the OptForce method to identify all genetic manipulations leading to the overproduction of succinate in *E. coli*[108]. The OptForce method consists of the following set of steps: define the constraints for both wild-type and mutant strains, perform flux variability analyses for both wild-type and mutant strains, find the sets of reactions that must alter their flux in order to achieve the desired phenotype in the mutant strain, and, finally, find the interventions needed to ensure an increased production of the target of interest (Steps 69–73).

First, select a commercial-grade solver and select the local directory to save the generated results with: >> changeCobraSolver(‘gurobi’, ‘ALL’);

57 | Load an illustrative model that comprises only 90 reactions, describing the central metabolism in *E. coli*[109].

```
>> readCbModel('AntCore.mat');
```

58 | Set the objective function to maximise the biomass reaction (R75). Change the lower bounds such that *E. coli* model will be able to consume glucose, oxygen, sulfate, ammonium, citrate, and glycerol.

```
>> model = changeObjective(model, 'R75', 1);

>> for rxn = {'EX_gluc', 'EX_o2', 'EX_so4', 'EX_nh3', 'EX_cit', 'EX_glyc'}

model = changeRxnBounds(model, rxn, −100, 'l');

end
```

59 | Define the constraints for both wild-type and mutant strains:

```
>> constrWT = struct('rxnList', {{'R75'}}, 'rxnValues', 14, 'rxnBoundType', 'b');
```

>> constrMT = struct('rxnList', {{'R75', 'EX_suc'}}, 'rxnValues', [0, 155.55], … 'rxnBoundType', 'bb');

▲ **CRITICAL STEP** In this example, we provide the constraints for both wild-type and mutant strains, but in a typical scenario the definition of differential constraints on wild-type and mutant strains requires additional research. This step could take a few days or weeks, depending on the information available for the species of interest. Flux bounds (i.e., uptake rate and minimum biomass yield target) are required inputs. New experiments might be required to be performed in addition to the literature curation task in order to obtain such data. Assumptions may also be made when describing the phenotypes of both strains, which will reduce the dependency on literature curation. It is important that the two strains are sufficiently different in order to be able to anticipate differences in reaction ranges.

60 | Performing flux variability analysis for both wild-type and mutant strains with:

>> [minFluxesW, maxFluxesW, minFluxesM, maxFluxesM] = FVAoptForce(model, constrWT, constrMT);

>> disp([minFluxesW, maxFluxesW, minFluxesM, maxFluxesM]);

61 | The MUST sets are the sets of reactions that must increase or decrease their flux in order to achieve the desired phenotype in the mutant strain. As shown in Figure 5, the first order MUST sets are MustU and MustL while second order MUST sets are denoted as MustUU, MustLL, and MustUL. After parameters and constraints are defined, the functions findMustL and findMustU are run to determine the mustU and mustL sets, respectively. Define an ID of the run with:

>> runID = 'TestoptForceM';

Each time the MUST sets are determined, folders are generated to read inputs and store outputs, i.e., reports. These folders are located in the directory defined by the uniquely defined runID.

62 | In order to find the first order MUST sets, constraints should be defined:

>> constrOpt = struct('rxnList', {{'EX_gluc', 'R75', 'EX_suc'}}, 'values', [−100; 0; 155.5]);

63 | The first order MUST set MustL is determined by running:

>> [mustLSet, pos_mustL] = findMustL(model, minFluxesW, maxFluxesW, …

' constrOpt', constrOpt, 'runID', runID);

If runID is set to 'TestoptForceL', a folder *TestoptForceL* is created, in which two additional folders *InputsMustL* and *OutputsMustL* are created. The *InputsMustL* folder contains all the inputs required to run the function findMustL, while the *OutputsMustL* folder contains the mustL set found and a report that summarises all the inputs and outputs. In order to maintain a chronological order of computational experiments, the report is timestamped.

64 | Display the reactions that belong to the mustL set using:

```
>> disp(mustLSet)
```

65 | The first order MUST set Mustu is determined by running:

```
>> [mustUSet, pos_mustU] = findMustU(model, minFluxesW, maxFluxesW, …

‘ constrOpt’, constrOpt, ‘runID’, runID);
```

The results are stored and available in a format analogous to the mustL set. The reactions that belong to the mustU may be displayed in the same way as mustL.

66 | Define the reactions that will be excluded from the analysis. The reactions found in the previous step as well as exchange reactions shall be included.

```
>> constrOpt = struct(‘rxnList’, {{‘EX_gluc’, ‘R75’, ‘EX_suc’}}, ‘values’, [−100, 0, 155.5]’);

>> exchangeRxns = model.rxns(cellfun(@isempty, strfind(model.rxns, ‘EX_’)) == 0);

>> excludedRxns = unique([mustuSet; mustLSet; exchangeRxns]);
```

67 | The second order MUST set Mustuu can be determined by running:

```
>> [mustUU, pos_mustUU, mustUU_linear, pos_mustUU_linear] = findMustUU(model, minFluxesW, … maxFluxesW, ‘constrOpt’, constrOpt, ...

‘excludedRxns’, excludedRxns,’runID’, runID);
```

The results are stored and available in a format analogous to the mustL set. The reactions of the mustUU can be displayed using the disp function.

68 | Repeat the above steps to determine the second order MUST sets MustLL and MustUL by using the functions findMustLL and findMustUL respectively. The results are stored and available in a format analogous to the mustL set. In the present example, mustLL and mustuL are empty sets. **?**
TROUBLESHOOTING

69 | In order to find the interventions needed to ensure an increased production of the target of interest, define the mustu set as the union of the reactions that must be up-regulated in the first and second order MUST sets. Similarly, mustL may be defined.

```
>> mustU = unique(union(mustUSet, mustUU));

>> mustL = unique(union(mustLSet, mustLL));
```

70 | Define the number of interventions κ allowed, the maximum number of sets to find nSets, the reaction producing the metabolite of interest targetRxn (in this case, succinate), and the constraints on the mutant strain constrOpt.

```
>> k = 1; nSets = 1; targetRxn = ‘EX_suc’;

>> constrOpt = struct(‘rxnList’, {{‘EX_gluc’,’R75’}}, ‘values’, [−100, 0]);
```

71 | Run the OptForce algorithm and display the reactions identified by optForce with:

```
>> [optForceSets, posoptForceSets, typeRegoptForceSets, flux_optForceSets] = …
optForce(model, targetRxn, mustU, mustL, minFluxesW, maxFluxesW, minFluxesM, ...
maxFluxesM, 'k', k, 'nSets', nSets, 'constrOpt', constrOpt, 'runID', runID);
```

72 | In order to find non-intuitive solutions, increase the number of interventions κ and exclude the SUCt reaction from up-regulations. Increase nSets to find the 20 best sets. Change the runID to save this second result in a separate folder from the previous result, then run optForce again as in Step 71.

```
>> k = 2; nSets = 20; runID = 'TestoptForceM2';
>> excludedRxns = struct('rxnList', {{'SUCt'}}, 'typeReg','U');
```

73 | The reactions determined by optForce can be displayed using disp(optForceSets). The complete set of predicted interventions can be found in the folders created inside the runID folder in which inputs and outputs of optForce and associated findMust* functions are stored. The input folders *InputsFindMust** contain *.mat* files for running the functions to solve each one of the bilevel optimisation problems. The output folders *OutputsFindMust** contain results of the algorithms (saved as *.xls* and *.txt* files) as well as a report (*a .txt* file) that summarises the outcome of the steps performed during the execution of each function. The optForce algorithm will find sets of reactions that should increase the production of a specified target. The first sets found should be the best ones because the production rate will be the highest. The last ones will be the worst, as the production rate is the lowest. **! CAUTION** Be aware that some sets may not guarantee a minimum production rate for a target, so check the minimum production rate, e.g., using the function testoptForceSol.

Atomically resolve a metabolic reconstruction ● TIMING 10 – 10^5 s

74 | In most genome-scale metabolic models, it is not explicit that the stoichiometric matrix represents a network of biochemical reactions. It is implicit that each row of the stoichiometric matrix corresponds to some molecular species, but when computing properties of the model, the atomic structure of each molecular species is not represented. It is also implicit that each column of the stoichiometric matrix corresponds to some biochemical reaction. However, when computing properties of the model, the mechanisms of the underlying biochemical reaction, in terms of the structures of the metabolites and the atomically resolved chemical transformations that take place, are not represented. Recent developments in genome-scale metabolic modelling have generated genome-scale metabolic reconstructions where the molecular structures are specified[110] and the reaction mechanisms are represented by atom mappings between substrate and product atoms[66, 111].

An atom mapping is a one-to-one association between a substrate atom and a product atom. An instance of a chemical reaction may be represented by a set of atom mappings, with one atom mapping between each substrate and product atom. A single chemical reaction can admit multiple chemically equivalent atom mappings when chemically equivalent atoms are present in a substrate, a product, or both. Therefore, each chemical reaction may be represented by one set, or multiple chemically equivalent sets, of atom mappings. Together, a set of atom mappings for a chemical reaction specify key aspects of the reaction mechanism, e.g., chemical bond change, breakage, and formation. The Virtual Metabolic Human database (VMH, http://vmh.life) provides metabolites chemical structures and atom mapped reactions for 9,610 reactions in Recon3D[66] and 4,831 metabolites from Recon3D[66] and the human gut microbiota[38]. Metabolite structures are provided in canonically ordered MOL and SMILES formats. Atom mapping data are provided in both RXN and SMILES formats. This explicit representation of metabolite and reaction structure offers the possibility of a broader range of biological, biomedical and biotechnological applications than with stoichiometry alone.

In order to obtain chemical structures for each metabolite, there are three main ways:

(A) Use chemoinformatics software tools

- **(i)** Suitable cheminformatics software tools[110] may be used to automatically obtain metabolite identifiers in metabolic network reconstructions and download the corresponding structure from a database.

(B) Manually interrogate metabolic databases

- **(i)** Databases such as VMH (http://vmh.life), PubChem[112], KEGG[113], ChEBI[114], LMSD[115], BioPath database[116], ChemSpider database[117], HMDB[118], etc provide chemical structures for metabolites in a network.

(C) Manually draw structures of metabolites

- **(i)** Based on chemical knowledge, one could manually draw structures of metabolites using tools such as ChemDraw (PerkinElmer, https://perkinelmer.com/ChemDraw).

75 | In order to obtain an atom mapping for a metabolic reaction, the reaction stoichiometry and the chemical structures of the corresponding metabolites must be available. To obtain atom mappings, there are three main options:

(A) Use software tools for prediction of atom mappings

- **(i)** A comparative study has been performed using Recon3D as a test case[111]. Due to its accuracy and availability, Reaction Decoder Tool (RDT[119]) is considered being the most suitable algorithm to atom map the reactions from a genome-scale metabolic network. Nevertheless, note that the Canonical Labelling for Clique Approximation (CLCA[120]) algorithm

can map reactions with explicit hydrogen atoms for fully protonated reactions, while RDT can only atom map reactions with implicit hydrogen atoms.

(B) Manually interrogate metabolic databases

(i) Databases such as BioPath[116] and KEGG RPAIR[121] disseminate manually curated atom mappings. VMH (http://vmh.life) also contains manually curated atom mappings for a subset of human metabolic reactions.

(C) Manually draw atom mappings

(i) Based on chemical knowledge, one could draw atom mappings using tools such as ChemDraw (PerkinElmer, https://perkinelmer.com/ChemDraw).

76 | Given a model structure and the directory containing the chemical structure files (molFileDir) in MDL MOL file format, RDT can be invoked to atom map a metabolic model using:

```
>> balancedRxns = obtainAtomMappingsRDT(model, molFileDir, outputDir, …
maxTime, standardiseRxn);
```

This function computes atom mapping data for the balanced and unbalanced reactions in the metabolic network and saves it in the outputDir directory. The optional maxTime parameter sets a runtime limit for atom mapping of a reaction. If standardiseRxn == 1, then atom mappings are also canonicalised, which is necessary in order to obtain a consistent interoperable set of atom mappings for certain applications, e.g., computation of conserved moieties in Step 77. The output balancedRxns contains the balanced atom mapped metabolic reactions. **? TROUBLESHOOTING**

77 | With a set of canonicalised atom mappings for a metabolic network, the set of linearly independent conserved moieties for a metabolic network can be identified[122]. Each of these conserved moieties corresponds to a molecular substructure (set of atoms in a subset of a molecule) whose structure remains invariant despite all the chemical transformations in a given network. A conserved moiety is a group of atoms that follow identical paths through metabolites in a metabolic network. Similarly to a vector in the (right) nullspace of a stoichiometric matrix that corresponds to a pathway (see Step 52), a conserved moiety corresponds to a vector in the left nullspace of a stoichiometric matrix. Metabolic networks are hypergraphs[123], while most moiety subnetwork are graphs. Therefore conserved moieties have both biochemical and mathematical significance and once computed, can be used for a wide variety of applications. Given a metabolic network of exclusively mass balanced reactions, one can identify conserved moieties by a graph theory analysis of its atom transition network[122].

First compute an atom transition network for a metabolic network using:

```
>> ATN = buildAtomTransitionNetwork(model, rxnfileDir);
```

where rxnfileDir is a directory containing only atom mapped files from balanced reactions, which can be obtained as explained in Step 76. The output atn is a structure with several fields: .A is a $p \times q$ sparse incidence matrix for the atom transition network, where p is the number of atoms and q is the number of atom transitions, .mets is a $p \times 1$ cell array of metabolite identifiers to link each atoms to its corresponding metabolites, .rxns is a $q \times 1$ cell array of reaction identifiers to link atom transitions to their corresponding reactions, and .elements is a p x 1 cell array of element symbols for atoms in .A.

▲ **CRITICAL STEP** All the RXN files needed to compute the atom transition network must be in a canonical format. This can be achieved by setting standardiseRxn = 1.

78 | In order to identify the conserved moieties in the metabolic network, invoke:

```
>> [L, M, moietyFormulas] = identifyConservedMoieties(model, ATN);
```

where L represents the conserved moieties in the metabolic network. That is, Lis an $m \times r$ matrix of r moiety vectors in the left null space of the stoichiometric matrix, M is the $u \times v$ incidence matrix of the moiety supergraph in which each connected component is a moiety graph, and moietyFormulas is an $m \times r$ cell array with chemical formulas of the computed moieties.

Thermodynamically constrain a metabolic model ● TIMING 1 – 10^3 s

79 | In flux balance analysis of genome-scale stoichiometric models of metabolism, the principal constraints are uptake or secretion rates, the steady state mass conservation assumption, and reaction directionality. The COBRA Toolbox extension *vonBertalanffy*[124] is a set of methods for integration of thermochemical data with constraint-based models[125–127] as well as application of thermodynamic laws to increase the physicochemical fidelity of constraint-based modelling predictions[128]. A full exposition of the method to thermodynamically constrain a genome-scale metabolic model is beyond the scope of this protocol. Therefore, only several key steps are highlighted.

Given a set of experimentally derived training_data on standard transformed Gibbs energies of formation, a state-of-the art quantitative estimation of standard Gibbs energy of formation for metabolites with similar chemical substructures can be obtained using an implementation of the component contribution method[127]. We assume that the input model has been anatomically resolved as described in Steps 74–78. Access to a compendium of stoichiometrically consistent metabolite structures[110, 122] is a prerequisite. The component contribution method is then invoked as follows:

```
>> model = componentContribution(model, training_data);
```

The model.DfG0 field gives the estimated standard Gibbs energy of formation for each metabolite in the model with model.DfG0_Uncertainty field expressing the uncertainty in these estimates, which is smaller for metabolites structurally related to metabolites in the training set. All thermodynamic estimates are given in units of kJ/mol.

80 | The standard Gibbs energy of formation for each metabolite must be transformed according to the environment of each compartment of the model[126], i.e., the temperature, pH, ionic strength and electrical potential specific to each compartment. Then the thermodynamic properties of reactions are estimated, given model.concMin and model.concMax where one can supply lower and upper bounds on compartment-specific metabolite concentrations (mol/L), which may be achieved with:

```
>> model = setupThermoModel(model, confidenceLevel);
```

In the output, field .DfGt0 of model gives the estimated standard transformed Gibbs energy of formation for each metabolite and .DrGt0 gives the estimated standard transformed Gibbs energy for each reaction. Subject to a confidenceLevel specified as an input, the upper and lower bounds on standard transformed Gibbs energy for each reaction are provided in .DrGtMin and .DrGtMax respectively.

▲ **CRITICAL STEP** In a multi-compartmental model, this step must be done for an entire network at once in order to ensure that thermodynamic potential differences, arising from differences in the environment between compartments, are properly taken into account. See[126] for a theoretical justification for this assertion.

81 | Reaction directionality may be quantitatively assigned based on the aforementioned thermodynamic estimates with:

```
>> [modelThermo, directions]= thermoConstrainFluxBounds(model, confidenceLevel, …
DrGt0_Uncertainty_Cutoff);
```

If model.DrGtMax(j)< 0, then the j^{th} reaction is assigned to be forward, and if model.DrGtMin(j)> 0 then the j^{th} reaction is assigned to be reverse, unless the uncertainty in estimation of standard transformed reaction Gibbs energy exceeds a specified cutoff (DrGt0_uncertainty_Cutoff). In this case, the qualitatively assigned reaction directionality, specified together by model.lb(j) and model.ub(j), takes precedence. The directions output provides a set of boolean vector fields that can be used to analyse the effect of qualitatively versus quantitatively assigning reaction directionality using thermochemical parameters.

82 | Thermodynamically constrained flux balance analysis may then be invoked by disallowing flux around stoichiometrically balanced cycles, also known as loops, using the allowLoops parameter to

optimizeCbModel with:

```
>> allowLoops = 0;
>> solution = optimizeCbModel(model, [], [], allowLoops);
```

The solution structure is the same as for flux balance analysis (see Problem (1)), except that this solution satisfies additional constraints that ensure the predicted steady state flux vector is thermodynamically feasible[129]. The solution satisfies energy conservation and the second law of thermodynamics[130].

Convert a flux balance model into a kinetic model ● TIMING 1 – 10^3 s

83 | In order to analyse biochemical networks at genome scale, systems biologists often use a linear optimisation technique called flux balance analysis (FBA). Linear approximation to known nonlinear biochemical reaction network function is sufficient to get biologically meaningful predictions in some situations. However, there are many biochemical processes where a linear approximation is insufficient, which motivates the quest for developing variational kinetic modelling[131–133]. Certain conditions are required to be met in order to generate a kinetic model that is internally consistent. First we describe those conditions, then we demonstrate how to ensure that they are met. Consider a biochemical network with m molecular species and n reversible reactions. We define forward and reverse *stoichiometric matrices*, $F, R \in \mathbb{Z}_+^{m \times n}$, respectively, where F_{ij} denotes the *stoichiometry* of the i^{th} molecular species in the j^{th} forward reaction and R_{ij} denotes the stoichiometry of the i^{th} molecular species in the j^{th} reverse reaction. We assume that the network of reactions is stoichiometrically consistent[69], that is, there exists at least one positive vector $l \in \mathbb{R}_{++}^m$ satisfying $(R-F)^T l = 0$. Equivalently, we require that *every reaction conserves mass.* The matrix $N := R - F$ represents net reaction stoichiometry and may be viewed as the incidence matrix of a directed hypergraph[123]. We assume that there are less molecular species than there are net reactions, that is $m \prec n$. We assume the cardinality of each row of F and R is at least one, and the cardinality of each column of $R - F$ is at least two. The matrices F and R are sparse and the particular sparsity pattern depends on the particular biochemical network being modelled. Moreover, we assume that rank($[F, R]$) = m, which is a requirement for kinetic consistency[134].

Compute a non-equilibrium kinetic steady state ● TIMING 1 – 10^3 s

84 | Let $c \in \mathbb{R}_{++}^m$ denote a variable vector of molecular species concentrations. Assuming constant nonnegative elementary kinetic parameters $k_f, k_r \in \mathbb{R}_+^n$, we assume *elementary reaction kinetics* for forward and reverse elementary reaction rates as $s(k_f, c) := \exp(\ln(k_f) + F^T \ln(c))$ and $r(k_r, c) := \exp(\ln(k_r) + R^T \ln(c))$, respectively, where $\exp(\cdot)$ and $\ln(\cdot)$ denote the respective component-wise functions[134, 135]. Then, the deterministic dynamical equation for time evolution of molecular species concenration is given by

$$\frac{dc}{dt} \equiv N(s(k_f, c) - r(k_r, c)) = N(\exp(\ln(k_f) + F^T \ln(c)) - \exp(\ln(k_r) + R^T \ln(c))) =: -f(c). \quad (5)$$

A vector c^* is a *steady state* if and only if it satisfies $f(c^*) = 0$, leading to the nonlinear system

$$f(x) = 0\,.$$

There are many algorithms that can handle this nonlinear system by minimising a nonlinear least-squares problem; however, particular features of this mapping, such as sparsity of stoichiometric matrices F and R and non-unique local zeros of mapping f, motivates the quest for developing several algorithms for efficient dealing with this nonlinear system. A particular class of such mappings, called duplomonotone mapping, was studied for biochemical networks[136] and three derivative-free algorithms for finding zeros of strongly duplomonotone mappings were introduced. Further, it is shown that the function $\|f(x)\|^2$ can be rewritten as a difference of two convex functions that is suitable to be minimised with DC programming methods[135]. Therefore, a DC algorithm and its acceleration with adding a line search technique were proposed for finding a stationary point of $\|f(x)\|^2$. Since the mapping f has locally non-unique solutions, it does not satisfy classical assumptions (e.g., nonsingularity of the Jacobian) for convergence theory. As a result, it was proved that the mapping satisfies the so-called Hölder metric subregularity assumption[137] and an adaptive Levenberg-Marquardt method was proposed to find a solution of this nonlinear system if the starting point is close enough to a solution. In order to guarantee the convergence of the Levenberg-Marquardt method with arbitrary starting point, it is combined with globalisation techniques such as line search or trust-region, which leads to computationally efficient algorithms. Note that a stationary point of $\|f(x)\|^2$ may not correspond to a solution x, such that $f(x)=0$, when $\nabla f(x) f(x)=0$ does not imply $f(x)=0$.

Compute a non-equilibirum kinetic steady state by running the function optimizeVKmodel. The mandatory inputs for computing steady states are a model vKModel containing F and R, the name of a solver to solve the nonlinear system, an initial point x0, and parameters for the considered solvers. For example, for specifying a solver, we write solver = 'LMTR';. Optional parameters for the selected algorithm may be given to optimizeVKmodel by the params struct as follows >> params.MaxNumIter = 1000; params.adaptive = 1; params.kin = kin;

Otherwise, the selected algorithm will be run with the default parameters assigned for each algorithm. Running the function optimizeVKmodel is done by typing

>> output = optimizeVKmodel(vKModel, solver, x0, params);

The output struct contains information related to the execution of the solver.

Compute a moiety conserved non-equilibrium kinetic steady state ● TIMING 1 – 10^3 s

85 | Let us note that a vector c^* is a steady state of the biochemical system if and only if

$$s\left(k_f, c^*\right) - r\left(k_r, c^*\right) \in \mathcal{N}(N),$$

where $\mathcal{N}(N)$ denotes the null space of N. Therefore, the set of steady states $\Omega = \left\{c \in \mathbb{R}^m_{++} | f(c) = 0\right\}$ is unchanged if the matrix N is replaced by a matrix $\bar{N}$ with the same kernel. Suppose that $\bar{N} \in \mathbb{Z}^{r \times n}$ is the submatrix of N whose rows are linearly independent; then rank($\bar{N}$) = rank(N)=: r. If one replaces N by and transforms (5) to logarithmic scale, and by letting $x := \ln(c) \in \mathbb{R}^m, k := \left[\ln\left(k_f\right)^T, \ln\left(k_r\right)^T\right]^T \in \mathbb{R}^{2n}$, then the right-hand side of (5) is equal to the function

$$\bar{f}(x) := [\bar{N}, -\bar{N}]\exp\left(k + [F, R]^T x\right), \quad (6)$$

where $[\cdot, \cdot]$ stands for the horizontal concatenation operator. Let $L \in \mathbb{R}^{m-r,m}$ denote a basis for the left nullspace of N, which implies $LN = 0$. We have rank(L) = $m - r$. We say that the system satisfies *moiety conservation* if for any initial concentration $c_0 \in \mathbb{R}^m_{++}$,

$$L\,c = L\,\exp(x) = l_0,$$

where $l_0 \in \mathbb{R}^m_{++}$. It is possible to compute L such that each corresponds to a structurally identifiable conserved moiety in a biochemical network[122]. The problem of finding the *moiety conserved steady state* of a biochemical reaction network is equivalent to solving the nonlinear system of equations

$$h(x) := \begin{pmatrix} \bar{f}(x) \\ L\exp(x) - l_0 \end{pmatrix} = 0. \quad (7)$$

Among algorithms mentioned in the previous section, the local and global Levenberg-Marquardt methods[137] are designed to compute either a solution of the nonlinear system (7) or a stationary point of the merit function $\frac{1}{2}\left\|h(x)\right\|^2$. The computation of a moiety conserved non-equilibrium kinetic steady state is made by running the optimizeVKmodel function in the same way as in previous section. A model vKModel containing F and R, L and l_0 is then passed to optimizeVKmodel together with the name of one of the Levenberg-Marquardt solvers.

Human metabolic network visualisation with ReconMap ● TIMING 1 – 10^2 s

86 | The visualisation of biochemical pathways is an important tool for biologically interpreting predictions generated by constraint-based models. It can be an invaluable aid for developing an understanding of the biological meaning implied by a prediction. Biochemical network maps permit the visual integration of model predictions with the underlying biochemical context. Patterns that are

very difficult to appreciate in a vector can often be much better appreciated by studying a generic map contextualised with model predictions. Genome-scale biochemical network visualisation is particularly demanding. No currently available software satisfies all of the requirements that might be desired for visualisation of predictions from genome-scale models. Automatic layout of genome-scale biochemical networks is insufficiently developed to generate an aesthetically pleasing map, yet manual layout of such maps is very labour intensive and there is no global reference coordinate system for such maps, so each human might layout a global map differently. Software applications for graph visualisation are often not suited to displaying metabolic hypergraphs[138]. Client-server software models have to trade off between highly interactive display of subsystem maps[139] and less interactive display of genome-scale maps[51]. An additional challenge with genome-scale models is that there is too much detail to visually appreciate if an entire genome-scale map is visualised at once, necessitating the application of techniques to dimensionally reduce the presentation, e.g., semantic zooming[140]. With these caveats in mind, we present a method for genome-scale visualisation of human metabolic network predictions using ReconMap 2.01[50], a manual layout of the reactions in the human metabolic reconstruction Recon 2.04[17], visualised with the Molecular Interaction NEtwoRk visualisation (MINERVA[51]), a stand-alone web service built on the Google Maps (Google Inc.) application programming interface, that enables low latency web display and navigation of genome-scale molecular interaction networks.

Visualisation of context-specific predictions in ReconMap via a web browser depends on access to a server running MINERVA, which requests user credentials for remote access. Public access to this server is provided free of charge. To request user credentials, navigate with a web browser to http://vmh.life/#reconmap, select ADMIN (bottom left), and click on *Request an account* to send an email to the MINERVA team and subsequently receive your user credentials.

87 | In order to prepare for remote access from within MATLAB, load the details of the MINERVA instance on the remote server, which are provided within the COBRA Toolbox during installation, then add your user credentials to it: >> load(‘minerva.mat’);

```
>> minerva.login = ‘username’;

>> minerva.password = ‘password’; minerva.map = ‘ReconMap–2.01’;
```

88 | Load a human metabolic model into MATLAB with:

```
>> model = readCbModel(‘Recon2.v04.mat’);
```

89 | Change the objective function to maximise ATP production through complex V (ATP synthase, ‘ATPS4m’) in the electron transport chain with:

```
>> modelATP = changeObjective(model, ‘ATPS4m’);
```

90 | Although the optimal objective value of the flux balance analysis Problem (1) is unique, the optimal flux vector itself is most likely not. When visualising a flux vector, it is important that a unique solution to some optimisation problem is displayed. For example, we can predict a unique network flux by regularising the flux balance analysis Problem (1) by redefining $\rho(v) := c^T v - \frac{\sigma}{2} v^T v$ and $\sigma = 10^{-6}$ (see Step 21). In order to obtain a unique optimal flux vector, run:

```
>> solution = optimizeCbModel(modelATP, 'max', 1e-6);
```

91 | Build the context-specific overlay of a flux vector on ReconMap by instructing the COBRA Toolbox to communicate with the remote MINERVA server using:

```
>> identifier = 'your_overlay_title';

>> response = buildFluxDistLayout(minerva, model, solution, identifier);
```

The only new input variable is the text string in the identifier that enables you to name each overlay according to a unique title. The response status will be set to 1 if the overlay was successfully received by the MINERVA server. **? TROUBLESHOOTING**

92 | Visualise context-specific ReconMaps using a web browser. Navigate to http://vmh.life/#reconmap, login with your credentials above then select 'OVERLAYS' and the list of USER-PROVIDED OVERLAYS appears. In order to see the map from Step 91, check the box adjacent to the unique text string provided by identifier.

93 | In order to export context-specific ReconMaps as publishable graphics, two options are possible: portable document format *(.pdf)* or portable network format *(.png)*. The former is useful for external editing whereas the latter essentially produces a snapshot of the visual part of the map.

(A) PDF export

(i) Zoom out until the entire map is visible. Right click on the map –> Export as image –> PDF. A file named *model.pdf* will be downloaded to the default directory of the browser. This PDF is a scalable network graphic, so optionally one can use a PDF editor to zoom in or crop the PDF as desired.

(B) PNG export

(i) Navigate and zoom until the desired region of the map is visible. Right click on the map –> Export as image –> PNG and a file named *model.png* will be downloaded to the default directory of the browser.

Variable scope visualisation of a network with Paint4Net ● TIMING 1 – 10^3 s

94 | During model validation or optimisation, visualisation of a small-scale fragment of the network area of interest is often sufficient and is especially convenient during network reconstruction when a manual layout may not yet be available.

Automatic generation of a hypergraph layout for a chosen subset of network can be achieved with the COBRA Toolbox extension Paint4Net[141]. A subset of a network may be visualised, and the directionality and the fluxes for selected reactions may be shown. Details on each reaction (ID, name and synonyms, and formula) and metabolite (ID, name and synonyms, and charged formula) pop-up when a cursor is placed over the corresponding item.

First compute a flux vector, e.g., with flux balance analysis, using:

```
>> FBAsolution = optimizeCbModel(model);
```

95 | Visualise a selected network fragment around a list of reactions in a model, contextualised using a flux vector flux, by running:

```
>> flux = FBAsolution.v;
```

```
>> involvedMets = draw_by_rxn(model, rxns, drawMap, direction, initialMet, excludeMets, flux);
```

The rxns input provides a selection of reactions of interest. The remaining inputs are optional and control the appearance of the automatic layout. For example, excludeMets provides a list of metabolites that may be excluded from the network visualisation, e.g., cofactors such as NAD and NADP.

96 | In order to visualise a model fragment with a specified radius around a specified metabolite of interest, such as 'etoh[c] ', run:

```
>> metAbbr = {'etoh[c]'};
```

```
>> [involvedRxns, involvedMets] = draw_by_met(model, metAbbr, 'true', 1, 'struc', {''}, flux);
```

Contributing to the COBRA Toolbox with MATLAB.devTools ● TIMING 1 – 30 s

97 | A comprehensive code base such as the COBRA Toolbox evolves constantly. The open-source community is very active, and collaborators submit their contributions frequently. The more a new feature or bug fix is interlinked with existing functions, the higher the risk of a new addition breaking instantly code that is heavily used on a daily basis. In order to decrease this risk, a continuous integration setup interlinked with the version control system *git* has been set up. A *git*-tracked repository is essentially a folder with code or other documents of which all incremental changes are tracked by date and user.

Any incremental changes to the code are called commits. The main advantage of *git* over other version control systems is the availability of branches. In simple terms, a branch contains a sequence of incremental changes to the code. A branch is also commonly referred to as a feature. Consequently, a contribution generally consists of several commits on a branch.

Contributing to the COBRA Toolbox is straightforward. As a contributor to the COBRA Toolbox is likely more familiar with MATLAB than with the internal mechanics of *git,* the

MATLAB.devTools (https://github.com/opencobra/MATLAB.devTools) have been developed specifically to contribute to a *git*-tracked repository located on the Github server. In Figure 6, an overview of the two online repositories as well as their local copies is given.

There are two ways of using the COBRA Toolbox, which depends on the type of user.

(A) A user of the COBRA Toolbox

- **(i)** The openCOBRA repository (https://github.com/opencobra/cobratoolbox) is a public repository that is read-only. Once the openCOBRA repository has been installed (as explained in Steps 1–3) in the folder *cobratoolbox,* all branches (including *master* and *develop*) are available locally. In the local folder *cobratoolbox,* the user has read and write access, but *cannot* push eventual changes back to the openCOBRA repository. It is the default and stable *master* branch only that should be used. The local copy located in the *cobratoolbox* directory can be updated (both branches).

(B) A contributor or a developer of the COBRA Toolbox

- **(i)** In order to make changes to the openCOBRA repository, or, in other words, contribute, you must obtain your own personal copy first. You must register on the Github website (https://github.com) in order to obtain a *username.* First, click on the button **FORK** at the top right corner of the official openCOBRA repository website (https://github.com/opencobra/cobratoolbox) in order to create a personal copy (or *fork*) with write and read access of the openCOBRA repository. This copy is accessible under https://github.com/<username>/cobratoolbox. These branches can be accessed by following the procedure [2] (see Step 99).

After initialisation of the MATLAB.devTools, the user and developer may have two folders: a *cobratoolbox* folder with the stable *master* branch checked out, and a *fork-cobratoolbox* folder with the *develop* branch checked out. Detailed instructions for troubleshooting and/or contributing to the COBRA Toolbox using the terminal (or shell) are provided in Supplementary Manual 3.

After the official openCOBRA version of the COBRA Toolbox has been installed, it is possible to install the MATLAB.devTools from within MATLAB:

```
>> installDevTools
```

With this command, the directory *MATLAB.devTools* is created next to the *cobratoolbox* installation directory. The MATLAB.devTools can also be installed from the terminal (or shell):

```
$ git clone git@github . com: opencobra/MATLAB.devTools
```

▲ **CRITICAL STEP** A working internet connection is required and *git* and *curl* must be installed. Installation instructions are provided on the main repository page of the MATLAB.devTools. A valid passphrase-less SSH key must be set in the Github account settings in order to contribute without entering a password while securely communicating with the Github server. **? TROUBLESHOOTING**

98 | The MATLAB.devTools are configured on the fly or whenever the configuration details are not present. The first time a user runs contribute, the personal repository (fork) is downloaded (cloned) into a new folder named *fork-cobratoolbox* at the location specified by the user. In this local folder, both *master* and *develop* branches exist, but it is the *develop* branch that is automatically selected (*checked out*). Any new contributions are derived from the *develop* branch.

Initialising a contribution using the MATLAB.devTools is straightforward. In MATLAB, type:

```
>> contribute % then select procedure [1]
```

If the MATLAB.devTools are already configured, procedure [1] updates the fork (if necessary) and initialises a new branch with a name requested during the process. Once the contribution is initialised, files can be added, modified or deleted in the folder *fork-cobratoolbox.* A contribution is successfully initialised when the user is presented with a brief summary of configuration details. Instructions on how to proceed are also provided.

▲ **CRITICAL STEP** The location of the fork must be specified as the root directory. There will be a warning issued if the path already contains another git-tracked repository. **? TROUBLESHOOTING**

99 | An existing contribution can be continued after a while. This step is particularly important in order to retrieve all changes that have been made to the openCOBRA repository in the meantime.

```
>> contribute % then select procedure [2]
```

Procedure [2] pulls all changes from the openCOBRA repository, and rebases the existing contribution. In other words, existing commits are shifted forward and placed after all commits made on the *develop* branch of the openCOBRA repository.

▲ **CRITICAL STEP** Before attempting to continue working on an existing feature, make sure that you published your commits as explained in Step 100. **? TROUBLESHOOTING**

100 | Publishing a contribution means uploading the incremental code changes to the fork. The changes are available in public, but not yet available in the openCOBRA repository. A contribution may only be accepted in the official repository once a pull request has been submitted. It is not necessary to open a pull request if you want to simply upload your contribution to your fork.

```
>> contribute % then select procedure [3]
```

When running procedure [3], you have two options:

(A) Simple contribution without opening a pull request

(i) All changes to the code are individually listed and the user is asked explicitly which changes shall be added to the commit. Once all changes have been added, a commit message must be entered. Upon confirmation, the changes are pushed to the online fork automatically.

(B) Publishing and opening a pull request

(i) The procedure for submitting a pull request is the same as Option (A) with the difference that when selecting to open a pull request, a link is provided that leads to a pre-configured website according to the contributing guidelines. The pull request is then one click away.

▲ **CRITICAL STEP** After following procedures [1] and [2], all changes should be published using procedure [3] before stopping to work on that contribution. When following procedure [3], the incremental changes are uploaded to the remote server. It is advised to publish often, and make small incremental changes to the code. There is no need for opening a pull request immediately (Option A) if there are more changes to be made. A pull request may be opened at any time, even manually and directly from the Github website. **? TROUBLESHOOTING**

101 | If a contribution has been merged into the *develop* branch of the openCOBRA repository (accepted pull request), the contribution (feature or branch) can be safely deleted both locally and remotely on the fork by running contribute and selecting procedure [4].

Note that deleting a contribution deletes all the changes that have been made on that feature (branch). It is not possible to selectively delete a commit using the MATLAB.devTools. Instead, create a new branch by following procedure [1] (see Step 98), and follow the instructions to *cherry-pick* in the Supplementary Manual 3.

▲ **CRITICAL STEP** Make sure that your changes are either merged or saved locally if you need them. Once procedure [4] is concluded, all changes on the deleted branch are removed, both locally and remotely. No commits can be recovered. **? TROUBLESHOOTING**

102 | It is sometimes useful to simply update the fork without starting a new contribution. The local fork can be updated using procedure [5] of the contribute menu.

>> contribute % then select procedure [5]

▲ **CRITICAL STEP** Before updating your fork, make sure that no changes are present in the local directory *fork-cobratoolbox*. You can do so by typing:

>> checkStatus

If there are changes listed, publish them by selecting procedure [3] of the contribute menu as explained in Step 100. **? TROUBLESHOOTING**

Engaging with the COBRA Toolbox forum ● TIMING 1 – 10^2 s

103 | The Frequently Asked Questions (FAQ) section of the documentation (https://opencobra.github.io/cobratoolbox/docs/FAQ.html) is a good starting point to find answers to questions or issues one may face.

The public forum associated with the COBRA Toolbox, available at https://groups.google.com/forum/#!forum/cobra-toolbox, is a great way to search for solutions to previously recognised problems that are similar to problems novel to the user. This is especially so with respect to recent installation and configuration issues that have arisen due to asynchronous development of the many software packages integrated with the COBRA Toolbox.

104 | Suggest new solutions to problems

(A) Post your question to the online COBRA Toolbox forum

Questions posted in the forum are welcome provided that some simple guidelines are followed:

(i) Before a question can be posted, an application for membership at https://groups.google.com/forum/#!forum/cobra-toolbox is required to eliminate spam.

(ii) Make the question as detailed as possible to increase the probability of a rapid and helpful reply.

(iii) Append your message with the result of running generateSystemConfigReport so that repository maintainers are aware of the system configuration. That is often the first question that comes to mind when considering to respond.

(B) Reply to a question online COBRA Toolbox forum

(i) Community contributions are welcomed to help users overcome any issues they face and are noticed by existing COBRA community members.

Generally, responses to questions can be expected within 1–2 days of posting, provided that posting guidelines are followed.

?TROUBLESHOOTING

Step	Problem	Possible reason	Solution
1 |	The initCobraToolbox function displays warnings or error messages.	Incompatible third-party software or improperly configured system.	First, read the output of the initialisation script in the command window. Any warning or error messages, though often brief, may point toward the source of the problem during initialisation if read literally. Second, verify that all software versions are supported and have been correctly installed, as described in the MATERIALS section. Third, ensure that you are using the latest version of the COBRA Toolbox, cf. Steps 97–102. Fourth, verify and test the COBRA Toolbox,

Step	Problem	Possible reason	Solution
			as described in Step 3. Finally, if nothing else works, consult the COBRA Toolbox forum, as described in Steps 103–104.
3 |	Some tests are listed as failed when running testAll.	Some third party dependencies are not properly installed or the system is improperly configured.	Verify that all required software has been correctly installed as described in the MATERIALS section. The specific test can then be run individually to determine the exact cause of the error. If the error can be fixed, try to use the MATLAB.devTools and contribute a fix. Further details on how to approach submitting a contribution are given in Steps 97–102. If the error cannot be determined, reach out to the community as explained in Steps 103–104.
4 |	The readCbModelfunction fails to import a model.	The input file is not correctly formatted or the SBML file format is not supported.	Specifications for Excel sheets accepted by the COBRA Toolbox can be found on Github (opencobra.github.io/cobratoolbox/docs/COBRAModelFields.html). Files with legacy SBML formats can be imported, but some information from the SBML file might be lost. In addition to constraint-based information encoded by fields of the *fbc* package, the COBRA-style annotations introduced in the COBRA Toolbox 2.0[4] are supported for backward compatibility. Some information is still stored in this type of annotations. The data specified with the latest version of the *fbc* package is used in preference to other fields, e.g., legacy COBRA-style notes which may contain similar data.
4 |	The readCbModel function fails to import a model saved as a .mat file	The model may contain deprecated fields or fields which have invalid values.	Old MATLAB models saved as .mat files sometimes contain deprecated fields or fields which have invalid values. Some of these instances are checked and corrected during readCbModel but there might be instances, where readCbModel fails. If this happens, it is advisable, to load the mat file, run the verifyModel function on the loaded model, and manually adjust all indicated inconsistent fields. After this procedure, we suggest to save the model again and use readCbModel to load the model.
4 |	The readCbModel function fails to import a SBML file	The model might be invalid	If an SBML file produces an error during IO, check that the file is valid SBML using the SBMLValidator (http://sbml.org/Facilities/Validator).
5 |	The writeCbModel function fails to export a model.	Some of the required fields of the model structure are missing or the model contains invalid data.	Before a reconstruction or model is exported, a summary of invalid data in the model can be obtained by running verifyModel(model). A list of required fields for the model structure is presented in Table 3.
15 |	The dqqMinos or quadMinos interfaces are not working as intended.	The binaries might not be compatible with your operating system.	Make sure that all relevant system requirements described in the MATERIALS section are satisfied. If you are still unable to use the respective interfaces, reach out to the community as explained in Steps 103–104.
16 | A)	The findSExRxnInd function fails to identify some exchange, demand and sink reactions.	Some exchange, demand and sink reactions do not start with any of anticipated prefixes.	Try an alternative approach.
16 | B)	The function checkMassChargeBalai returns wrong results.	Some formulae are icmissing or a formula is incorrectly specified, leading to one or more reactions to be incorrectly identified as being elementally balanced.	Try an alternative approach.

Step	Problem	Possible reason	Solution
16 | C)	Erroneous predictions.	Inadvertent violation of the steady-state mass conservation constraint.	Manually inspect the reaction formulae for each reaction to identify any obviously mass imbalanced reactions, omit them from the reconstruction and run findStoichConsistentSubset again.
22 |	The solution status given by FBAsolution.stat is −1.	A too short runtime limit has been set or numerical issues happened during the optimisation procedure.	Check the value of FBAsolution. origStat and compare it with the documentation provided by the solver in use for further information. If one is solving with a double precision solver a model that could be multi-scale but is not yet recognised as such, then FBAsolution.stat == −1 can be symptomatic of this situation. In that case, refer to Steps 14–15 to learn how to numerically characterise a reconstruction model.
55 |	The sampling distribution is not uniform (revealed by a non-uniform marginal flux distribution).	The sampling parameters options.nSkip and options.nSamples are set too low.	Increase the values of the options.nSkip and options.nSamples parameters until smooth and unimodal marginal flux distributions are obtained.
68 |	No reaction is found in the MUST sets.	The wild-type or mutant strain may not be enough constrained.	A solution is to add more constraints to the strains until differences in the reaction ranges are shown. If no differences are found, another algorithm might be better suited. If there is an error when running the findMust* functions, a possible reason is that the inputs are not well defined or a solver may not be set. Verify the inputs, use changeCobraSolver to change to a commercial-grade optimisation solver (see Table 4 for a list of supported solvers).
76 |	Some reactions could not be mapped.	Too short runtime limit or a reaction that the algorithm could not atom map.	Increase the runtime limit of the algorithm.
91 |	The remote MINERVA server refuses to build a new overlay.	The text string in the identifier input variable is not uniquely defined in your account.	Change the identifier text string of your overlay.
98 |	An error occurs when run running contribute claiming that the fork cannot be reached or that the local fork cannot be found.	The local forked folder cannot be found, has been moved, or the remote fork cannot be reached.	It may occur that the configuration of the MATLAB.devTools is faulty or has been mistyped. In that case, try to reset the configuration by typing: >> resetDevTools
97 |	An error might be thrown claiming permission denied.	The SSH key of the computer is not configured properly.	The installation of the MATLAB.devTools is dependent on a correctly configured Github account. The SSH key of the computer must be set in the Github account settings or otherwise errors will be thrown. If the *git clone* command works, the SSH key is properly set. In that case, delete the SSH key locally (generally located in the folder *.ssh* in the home directory) and remotely on Github, and generate a new SSH key.
98 |	Procedure [1] when running contribute might not be successful.	The local *fork-cobratoolbox* folder is too old or has not been updated for a while.	In that case and if no local changes are present, backup and remove the local *fork-cobratoolbox* folder and run the contribute command again. Alternatively, try to delete the forked repository online and re-fork the openCOBRA repository. When one is sure that everything is fine, the backup can be safely deleted, but it is wise to store it for some time, in case later one realises that some updates to code have gone missing.
98 |	Procedure [1] when running contribute might not be successful.	There are changes in the local *fork-cobratoolbox* folder.	Contribute the changes manually as described in the Supplementary Manual 3.

Step	Problem	Possible reason	Solution
98 |	Procedure [1] when running contribute might not be successful.	The forked repository cannot be reached online or the SSH key is not configured properly.	Set the SSH key in your Github account and make sure that the forked repository can be reached. This can easily be checked by re-cloning the MATLAB.devTools in the terminal as explained in Steps 97 and by browsing to the forked repository online.
99 |	Procedure [2] when running contribute might fail.	Your contribution has been deleted online, or is no longer available locally.	When the rebase process fails, the user is asked to reset the contribution, which will reset the contribution to the online version of the branch in the fork. In general, when the rebase fails there have been changes made on the openCOBRA repository that are in conflict with the local changes. You can check the status of the local repository by typing: >> checkStatus If there are conflicts that you do not know how to resolve, check the official repository or ping the developers in https://groups.google.com/forum/#!forum/cobra-toolbox as explained in Steps 103–104. If you already have published changes, try to submit a pull request as explained in Step 100 for developers to understand the situation. Alternatively, you can try to resolve the conflicts manually. More information on how to solve conflicts is given as Supplementary Manual 3.
100 |	Procedure [3] when running contribute might not be successful.	The forked repository cannot be reached online or if the SSH key is not configured properly.	Check to set the SSH key in your Github account and make sure that the forked repository can be reached.
100 |	When opening a pull request, Github cannot automatically merge.	There have been changes made on the openCOBRA repository and on your local fork.	Submit however the pull request; another developer will help you rebase your contribution manually.
101 |	Procedure [4] when running contribute might not be successful.	Your local changes are not yet published (committed).	Follow procedure [3] of the contribute menu in order to publish your changes first as explained in Step 100.
102 |	Procedure [5] when running contribute might not be successful.	There are some local changes that have not yet been published (committed).	Backup eventual modifications, remove the *fork-cobratoolbox folder*, and run the contribute command again.
102 |	Procedure [5] when running contribute might not be successful.	Too many changes have been made in the openCOBRA repository.	Backup your modified files to a separate location, and reset your branch manually by typing in the terminal (be careful - this will delete all your changes locally, but not remotely): $ git reset --hard origin/<yourBranch> Then, copy your file back into the *fork-cobratoolbox* folder and contribute normally.

● TIMING

Steps 1–2, Initialisation of the COBRA Toolbox: 5 – 30 s

Step 3, Verify and test the COBRA Toolbox: ~ 10^3 s

Step 4, Importing a reconstruction or model: 10 – 10^2 s

Step 5, Exporting a reconstruction or model: 10 – 10^2 s

Step 6, Use of rBioNet to add reactions to a reconstruction: 1 – 10^3 s

Steps 7–8, Use of a spreadsheet to add reactions to a reconstruction: 1 – 10^3 s

Steps 9–13, Use of scripts with reconstruction functions: 1 – 10^2 s

Step 14, Check the scaling of a reconstruction : 1 – 10^2 s

Step 15, Select a double- or quad-precision optimisation solver: 1 – 5 s

Step 16, Identify stoichiometrically consistent and inconsistent reactions: 1 – 10^5 s

Step 17, Identify stoichiometrically consistent and inconsistent molecular species: 1 – 10^3 s

Step 18, Set simulation constraints: 1 – 10^3 s

Step 19, Identify molecular species that leak, or siphon, across the boundary of the model: 1 – 10^3 s

Step 20, Identify flux inconsistent reactions: 1 – 10^3 s

Steps 21–22, Flux balance analysis: 1 – 10^2 s

Step 23, Relaxed flux balance analysis: 1 – 10^3 s

Step 24, Sparse flux balance analysis: 1 – 10^3 s

Steps 25–27, Identify dead-end metabolites and blocked reactions: ~10^2 s

Steps 28–30, Gap fill a metabolic network: 10^2 – 10^5 s

Steps 31–33, Extracellular metabolomic data: 10^3 – 10^5 s

Steps 34–39, Intracellular metabolomic data: 10^2 – 10^4 s

Step 40, Integration of transcriptomic and proteomic data: 10^2 – 10^4 s

Steps 41–46, Adding biological constraints to a flux balance model: ~ 10^2 s

Steps 47–48, Qualitative chemical and biochemical fidelity testing: 10^2 – 10^3 s

Steps 49–51, Quantitative biochemical fidelity testing: 10^2 – 10^3 s

Step 52, MinSpan Pathways: a sparse basis of the nullspace of a stoichiometric matrix: 10^2 – 10^4 s

Step 53, Low dimensional flux variability analysis: 1 – 10^3 s

Step 54, High dimensional flux variability analysis: 1 – 10^5 s

Step 55, Uniform sampling of steady-state fluxes: 1 – 10^3 s

Steps 56–73, Identify all genetic manipulations leading to targeted overproductions: 10 – 10^5 s

Steps 74–78, Atomically resolve a metabolic reconstruction: 10 – 10^5 s

Steps 79–82, Thermodynamically constrain a metabolic model: 1 – 10^3 s

Step 83, Convert a flux balance model into a kinetic model: 1 – 10^3 s

Step 84, Compute a non-equilibrium kinetic steady state: 1 – 10^3 s

Step 85, Compute a moiety conserved non-equilibrium kinetic steady state: 1 – 10^3 s

Steps 86–93, Human metabolic network visualisation with ReconMap: 1 – 10^2 s

Steps 94–96, Variable scope visualisation of a network with Paint4Net: 1 – 10^3 s

Steps 97–102, Contributing to the COBRA Toolbox with MATLAB.devTools: 1 – 30 s

ANTICIPATED RESULTS

Initialisation of the COBRA Toolbox

1 | The initialisation step automatically checks the configuration of all of the required and some of the optional software dependencies. During initialisation, all *git* submodules are updated. The solver paths are set when available and compatible. A system-dependent table with the solver status is returned, together with solver suggestions as shown in Figure 7. The user is also presented with options to update the COBRA Toolbox.

2 | A list of solvers assigned to solve each class of optimisation solvers is returned:

Defined solvers are:

CBT_LP_SOLVER: gurobi

CBT_MILP_SOLVER: gurobi

CBT_QP_SOLVER: qpng

CBT_MIQP_SOLVER: gurobi

CBT_NLP_SOLVER: matlab

3 | The test suite starts by initialising the COBRA Toolbox and thereafter, all of the tests are run. At the end of the test run, a comprehensive summary table is presented in which the respective tests and their test outcome is shown. On a fully configured system that is compatible with the most recent version of the COBRA Toolbox, all tests should pass. It may not be necessary to have a fully configured system to use one's particular subset of methods.

Importing a reconstruction or model

4 | The reconstruction or model is loaded into the MATLAB workspace within a structure named model, irrespective of whether the fileName specified a reconstruction or model. The model structure should contain all of the information in different fields. Table 3 provides an overview of the individual

model fields and their content. Very large SBML models may take some time to load.

5 | An exported file containing the information from the model in the location and format specified by the fileName.

Check the scaling of a reconstruction

14 | The checkScaling function returns a precisionEstimate string that is either 'double' or 'quad'. The scaling estimate is based on the order of magnitude of the ratio of the maximum and minimum row and column scaling coefficients, which are determined such that the scaled stoichiometric matrix has entries close to unity. In addition, a summary of scaling properties included in scalingProperties may be returned.

Select a double- or quad-precision optimisation solver

15 | If the selected solver is installed, solverStatus == 1 will be returned, the solver interface to MATLAB is configured correctly, and the solver is compatible with the system environment. If the dqqMinos solver has been selected and solverStatus == 1, then LP problem solutions are computed somewhat slower than with a double precision solver, but with the advantage that solutions are computed with a feasibility and optimality tolerance of 10^{-15}, which becomes an advantage for a multi-scale model, where the typical tolerance of 10^{-6} for a double precision solver may be insufficient.

Identify stoichiometrically consistent and inconsistent molecular species

17 | Any molecular species corresponding to a non-zero entry within SConsistentMetBool is always involved in mass imbalanced reactions indicating that the stoichiometry is misspecified. Double check the chemical formulae involved in the corresponding reactions to ensure that, e.g., the stoichiometry for protons, cofactors, etc., leads to balanced reactions.

20 | Any non-zero entry in fluxinConsistentRxnBool indicates a flux inconsistent reaction, i.e., a reaction that does not admit a non-zero flux. Blocked reactions can be resolved by manual reconstruction[6], algorithmic reconstruction[82], or a combination of both.

Sparse flux balance analysis

24 | There should be no such cycle in a network with bounds that are sufficiently constrained. Figure 8 illustrates the cycle obtained from Recon3D with all internal reaction bounds set to zero.

Integration of transcriptomic and proteomic data

40 | createTissuespecificModel returns a COBRA model which is constrained with the context of the data provided to it. Usually, this means enrichment of reactions with high expression and omission of reactions with low expression

profiles. Each method returns a flux consistent model, hence it is likely that certain reactions, without experimental evidence, are added to the context-specific model in order to enable non-zero net flux through reactions for which supporting experimental evidence for activity exists.

Quantitative biochemical fidelity testing

51 | For the Recon3Dmodel, the anticipated yield is 32 ATP per unit of glucose, which compares favourably to the ATP yield of 31 ATP obtained from the biochemical literature.

Uniform sampling of steady-state fluxes

55 | The marginal flux distributions for each reaction should be smooth and uni-modal for any biochemical network with feasible set $\Omega: = \left\{ v | S_v = 0; l \leq v \leq u \right\}$.

Identify all genetic manipulations leading to targeted overproductions

71 | The identified reaction is suet, i.e., a transporter for succinate (an intuitive solution). However, changing the parameters will enable optForce to find non-intuitive solutions.

Identify all genetic manipulations leading to targeted overproductions

73 | Figure 9 illustrates the interventions predicted by the OptForce method for succinate overproduction in the AntCore *E. coli* model under aerobic conditions.

Thermodynamically constrain a metabolic model

82 | Thermodynamically constrained flux predictions can differ markedly from those obtained with flux balance analysis. An open challenge is acquisition of sufficient thermochemical training data as well as sufficient quantitative metabolomic data, such that estimates of transformed reaction Gibbs energies can be made with sufficiently low uncertainty to constrain reaction directionality with high confidence. The degree of confidence typically differs markedly between reactions. Therefore, a pragmatic approach to rank order reaction directionality assignments by the probability that the thermodynamically assigned reaction directionality is forward, reversible or reverse (see Figure 10 for an application to Recon3D).

Human metabolic network visualisation with ReconMap

93 | Figure 11 illustrates the overlay of an optimal regularised flux balance analysis solution overlain within ReconMap within a web browser window.

Variable scope visualisation of a network with Paint4Net

96 | Figure 12 illustrates a fragment of a Paint4Net visualisation contextualised using a flux vector to control the thickness and colour of edges representing reactions. In the visualisation, one can discover the isolated parts of network without any flux, as well as cycles running without any substrate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

AUTHOR CONTRIBUTIONS

Author	Contributions
Sylvain Arreckx	Continuous integration, code review, opencobra.github.io/cobratoolbox, Jenkins, Documenter.py, changeCobraSolver, pull request support, tutorials, tests, coordination, manuscript, initCobraToolbox.
Laurent Heirendt	Continuous integration, code review, fastFVA (new version, test & integration), MATLAB.devTools, opencobra.github.io, tutorials, tests, pull request support, coordination, manuscript, initCobraToolbox, forum support.
Thomas Pfau	IO and transcriptomic integration, tutorials, tutorial reviews, IO and transcriptomic integration sections of manuscript, forum support, pull request support, code review.
Sebastián N. Mendoza	Development and update of strain design algorithms, GAMS and MATLAB integration, tutorials.
Anne Richelle	Transcriptomic data integration methods, tutorials, transcriptomic integration section of manuscript, RuMBA, pFBA, metabolic tasks, tutorial review.
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Hulda S. Haraldsdóttir	Thermodynamics, conserved moiety and sampling methods.
Jacek Wachowiak	Documentation.
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Vanja Vlasov	Tutorials.
Stefania Magnusdóttir	Multispecies modelling, tutorial review, testing.
Chiam Yu Ng	Strain design code review, tutorial review, manuscript (OptForce/biotech introduction).
German Preciat	Tutorials and chemoinformatics for metabolite structures and atom mapping data.
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John T. Sauls	modelBorgifier code and tutorial.
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Benjamin Cousins	CHRR uniform sampling.
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Luis V. Valcarcel	Tutorials and genetic MCSs implementation.
Iñigo Apaolaza	Tutorials and genetic MCSs implementation.
Susan Ghaderi	Interoperability with CellNetAnalyzer.
Masoud Ahookhosh	Adaptive Levenberg-Marquardt solver.
Marouen Ben Guebila	Tutorial reviews.
Andrejs Kostromins	Paint4Net code and tutorial.
Nicolas Sompairac	Development of metabolomic cartography tool and tutorial.
Hoai M. Le	Cardinality optimisation solver.
Ding Ma	Quad precision solvers.
Yuekai Sun	Multiscale flux balance analysis reformulation.

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Lin Wang	Strain design code review, tutorial review, manuscript (OptForce).
James T. Yurkovich	uFBA method and tutorial.
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Inna Kuperstein	Development of metabolomic cartography tool and tutorial.
Andrei Zinovyev	Development of metabolomic cartography tool and tutorial.
H. Scott Hinton	Tutorials.
William A. Bryant	Code refinement.
Francisco J. Aragón Artacho	Duplomonotone equation solver, boosted difference of convex optimisation solver, adaptive Levenberg-Marquardt solvers.
Francisco J. Planes	Academic Supervision, tutorials and genetic MCSs implementation.
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Ines Thiele	Academic supervision, tutorials, code contribution, manuscript.
Ronan M.T. Fleming	Conceptualisation, lead developer, academic supervision, software architecture, code review, sparse optimisation, nullspace computation, thermodynamics, variational kinetics, fastGapFill, sampling, conserved moieties, network visualisation, forum support, tutorials, manuscript.

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[155]. Boele J, Olivier BG, and Teusink B FAME, the Flux Analysis and Modeling Environment. BMC Systems Biology 6, 8 (2012). [PubMed: 22289213]

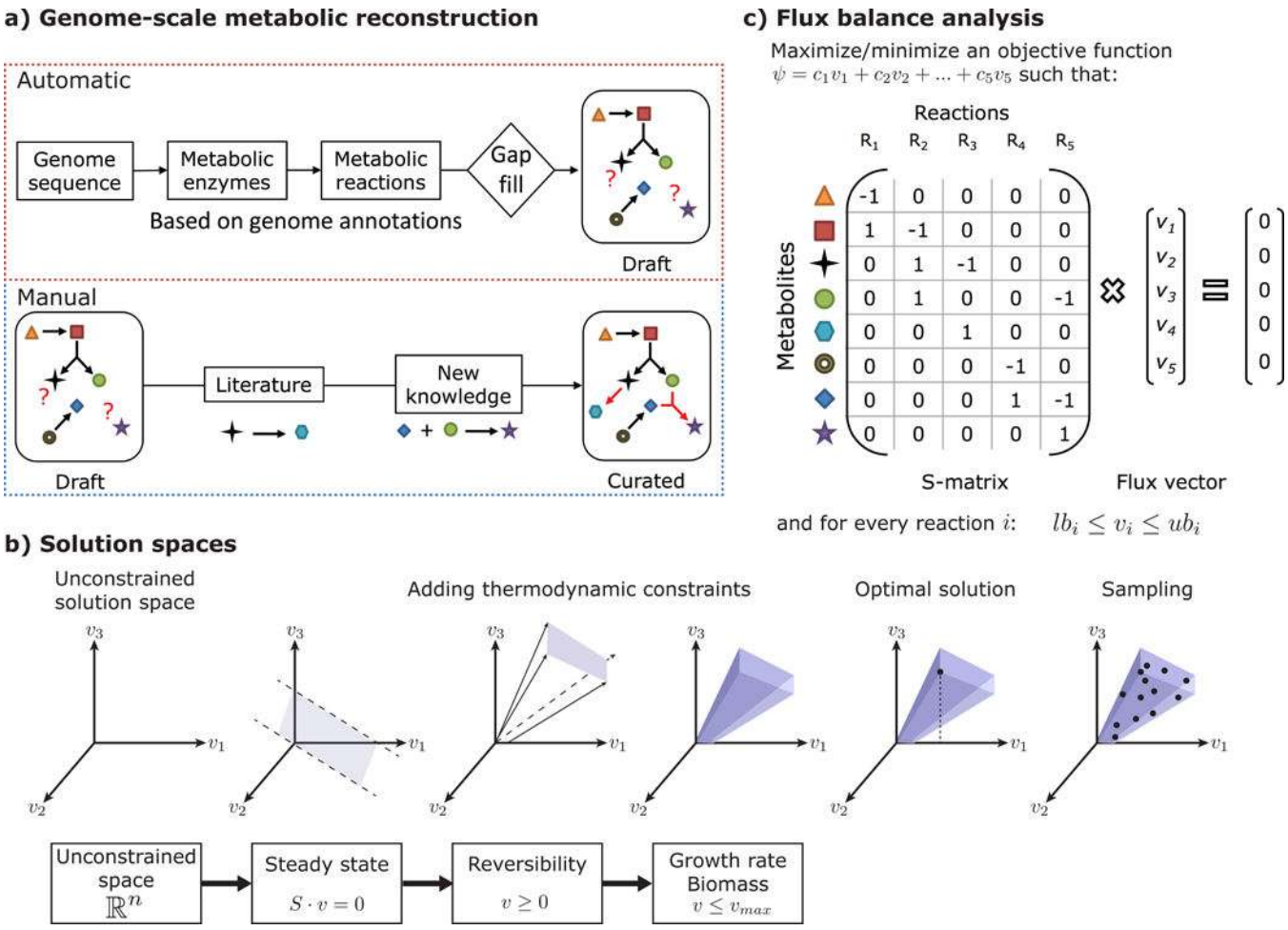

Figure 1:

Overview of key constraint-based reconstruction and analysis concepts. **a.** A genome-scale metabolic reconstruction is a structured knowledge-base that abstracts pertinent information on the biochemical transformations taking place within a chosen biochemical system, e.g., the human gut microbiome[38]. Genome-scale metabolic reconstructions are built in two steps. First, several platforms exist for the generation of a draft metabolic reconstruction based on genome annotations. Second, the draft reconstructions need to be refined based on known experimental and biochemical data from literature[6]. Novel experiments can be performed on the organism and the reconstruction refined accordingly. **b.** A phenotypically feasible solution space is defined by specifying certain assumptions, e.g., a steady-state assumption, then converting the reconstruction into computational model that eliminates physicochemically or biochemically infeasible network states. Various methods are used to interrogate the solution space. For example, optimisation for a biologically motivated objective function (e.g. biomass production) identifies a single optimal flux vector, whereas uniform sampling provides an unbiased characterisation via flux vectors uniformly distributed in the solution space. **c** Flux balance analysis is an optimization method that maximizes a linear objective function, $\psi(\upsilon) = c^T\upsilon$, formed by multiplying every reaction flux

v_j with a predetermined coefficient, c_j, subject to a steady state assumption, $S_v = 0$, as well as lower and upper bounds on each reaction flux (lb_j and ub_j, respectively).

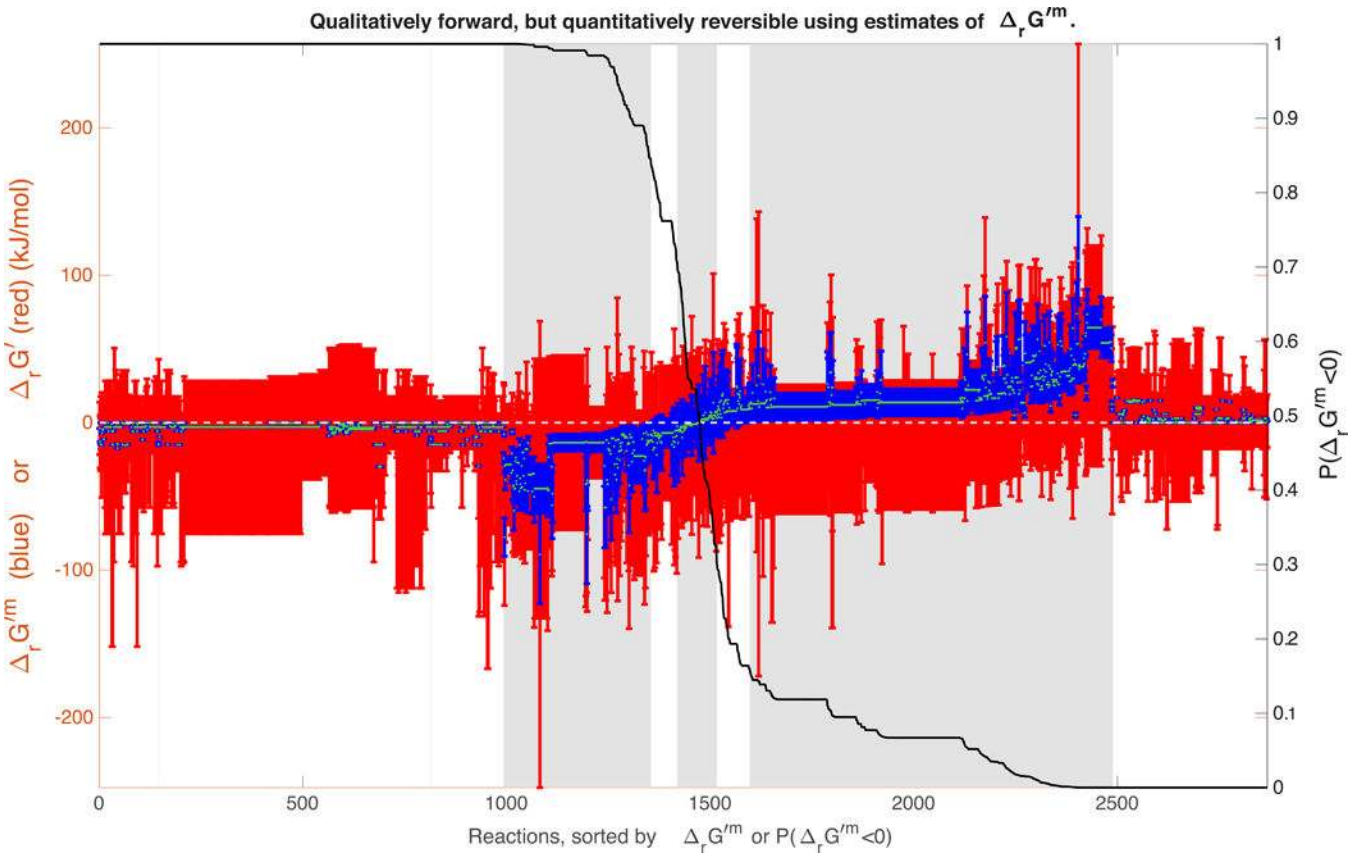

Figure 2:

Continuous integration of new code (submitted by developers) is performed on a dedicated server running Jenkins (https://jenkins.io). The main code is located in the *src* folder and tests functions in the *test* folder. A test not only runs a function (first degree testing), but tests the output of that function (second degree testing). The continuous integration setup relies on end-of-year releases of MATLAB only. Soon after the latest stable version of MATLAB is released, full support will be provided for the COBRA Toolbox. After a successful run of tests on the three latest end-of-year releases of MATLAB using various solver packages, the documentation based on the headers of the functions (docstrings) is extracted, generated, and automatically deployed. Immediate feedback through code coverage reports (https://codecov.io/gh/opencobra/cobratoolbox) and build statuses are reported on GitHub. With this setup, the impact of local changes in the code base is promptly revealed.

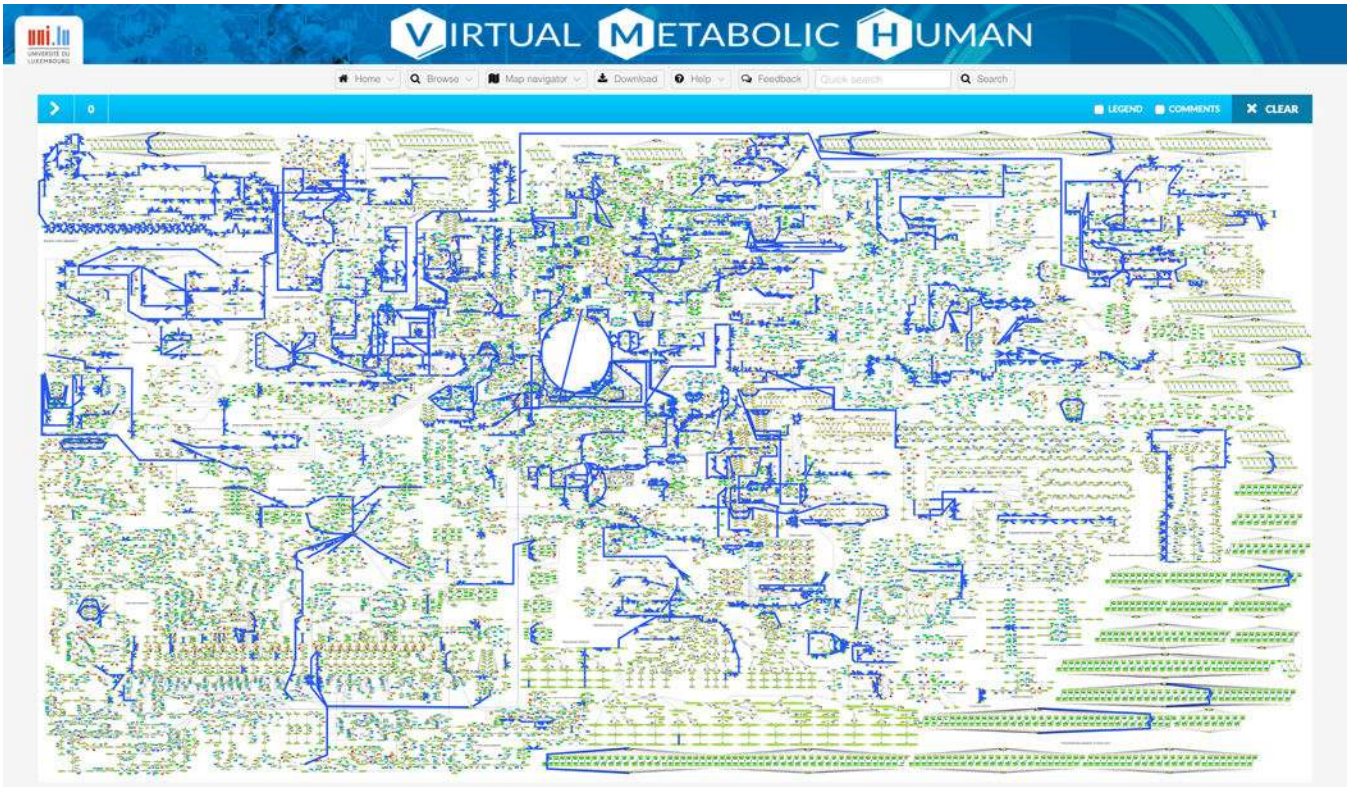

Figure 3:
Conceptual overview of the main steps involved in the unsteady-state flux balance analysis (uFBA) method.

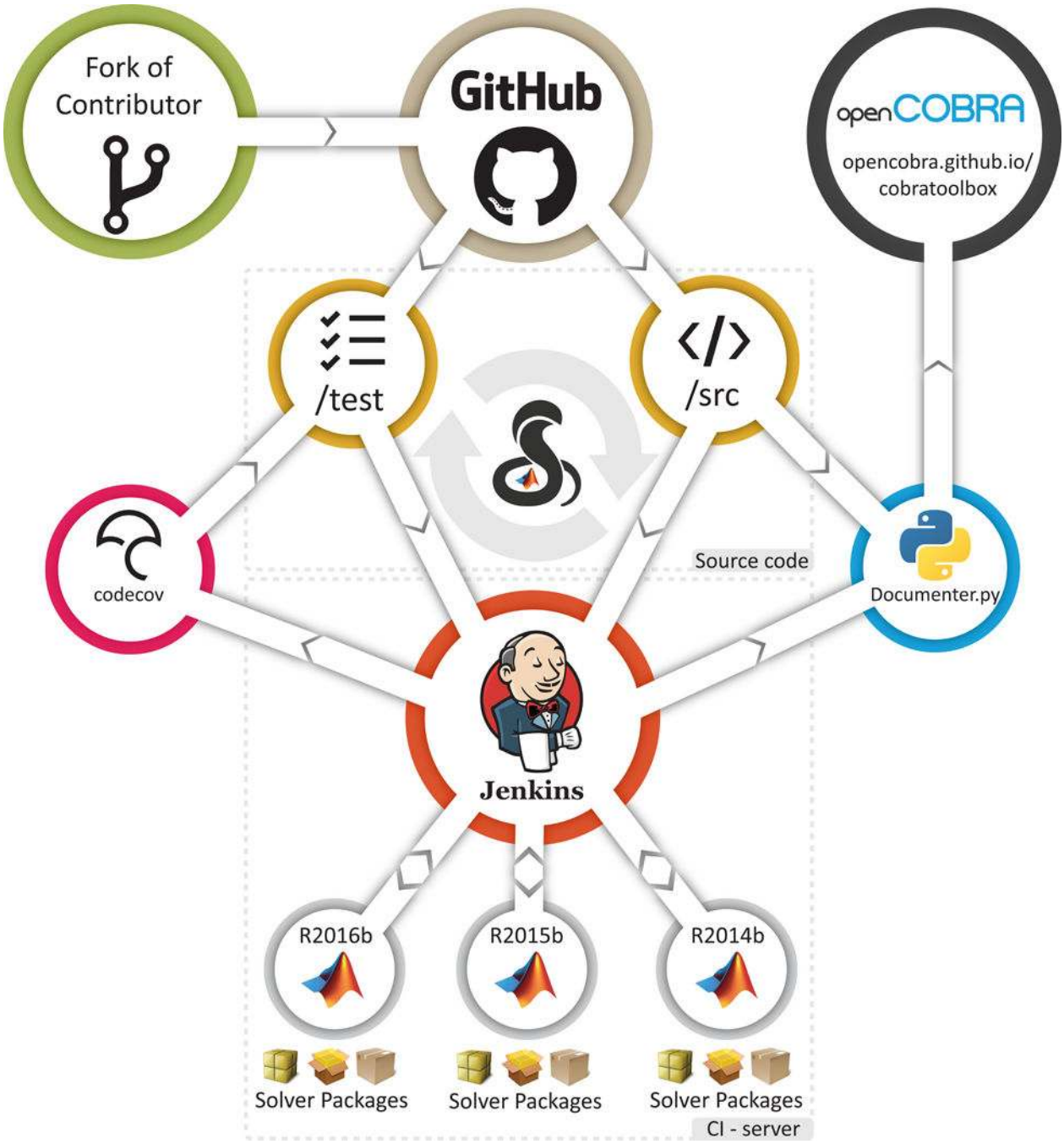

Figure 4:

Solution spaces from steady state fluxes are anisotropic, that is, long in some directions and short in others. This impedes the ability of any sampling algorithm taking a random direction to evenly explore the full feasible set (*artificial centering hit-and-run* (ACHR) algorithm). The CHRR (*coordinate hit-and-run with rounding*) algorithm first rounds the solution space based on the maximum volume ellipsoid. Then, the rounded solution space is uniformly sampled using a provably efficient coordinate hit-and-run random walk. Finally, the samples are projected back onto the anisotropic feasible set. This leads to a more

distributed uniform sampling, so that the converged sampling distributions for the selected reactions become smoother. As an example, for both sampling distributions, the parameters were defined as: $nSkip = 8 \times (dim(fluxSpace))^2$, $nSarnples = 1000$.

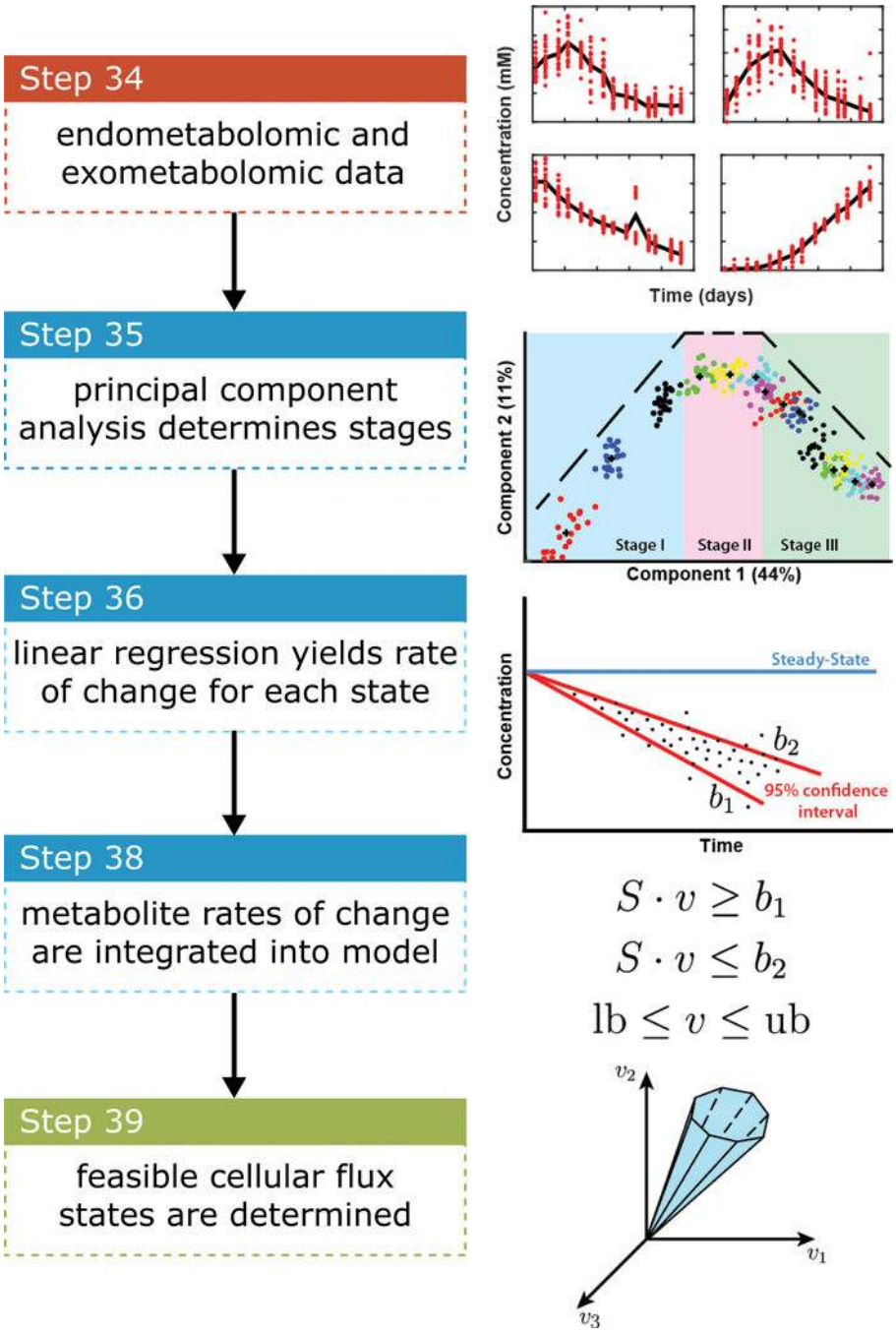

Figure 5:

In the OptForce procedure, the MUST sets are determined by contrasting the flux ranges obtained using flux variability analysis (FVA) of a wild-type (blue bars) and an overproducing strain (red bars). The first order MUST sets (top panel) are denoted $\mathrm{MUST}^{\mathrm{L}}$ and $\mathrm{MUST}^{\mathrm{U}}$. For instance, a reaction belongs to the $\mathrm{MUST}^{\mathrm{U}}$ set if the upper bound of the flux range in the wild-type is less than the lower bound of the flux range of the overproducing strain. The center and bottom panels show all possible second order MUST sets.

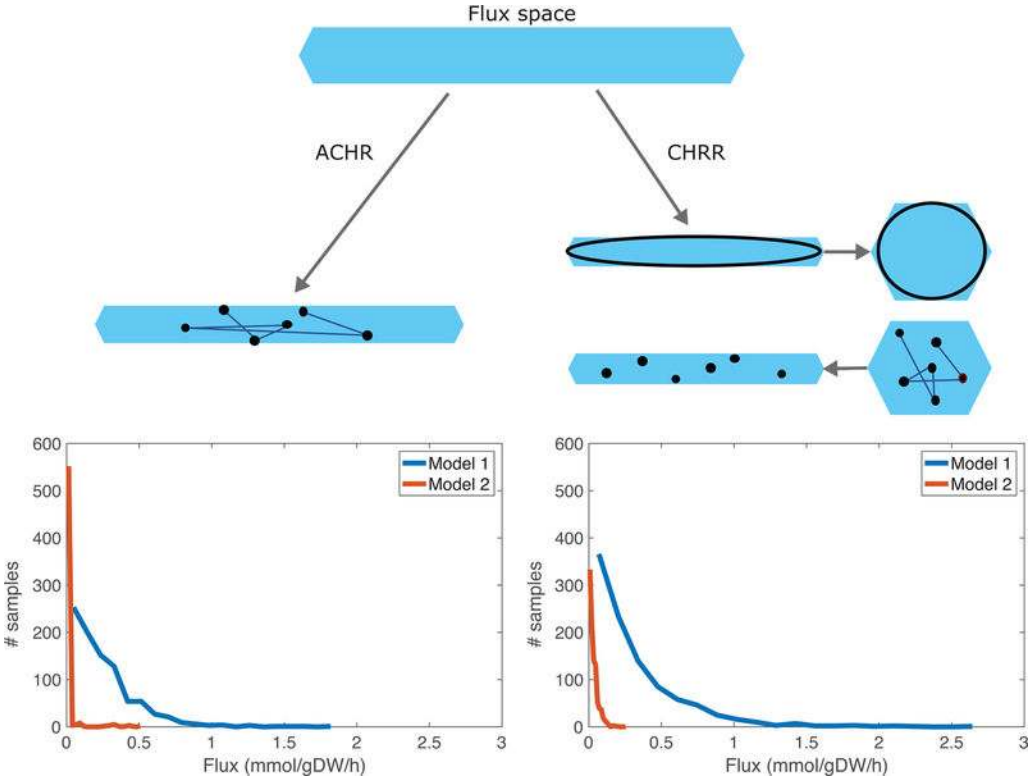

Figure 6:

The openCOBRA repository and the fork of a contributor located on the Github server can be cloned to the local computer as *cobratoolbox* and *fork-cobratoolbox* folders, respectively. Each repository might contain different branches, but each repository contains the *master* and *develop* branches. Note that contributors only have read on the openCOBRA repository. The stable branch is the *master* branch (black branch), while the development of code is made on the *develop* branch (green branch). The *master* branch shall be checked out when using the *cobratoolbox* repository, whereas contributors shall create new branches originating from the *develop* branch (local *fork-cobratoolbox* directory and online *<username>/cobratoolbox* repository). In the present example, *myBranch1* (blue branch) has already been pushed to the forked repository on the Github server, while *myBranch2* (pink branch) is only present locally. The branch *myBranch1* may be merged into the *develop* branch of the openCOBRA repository through opening a pull request. In order to submit the contributions (commits) on *myBranch2,* the contributor must first push the commits to the forked repository (https://github.com/<username>/cobratoolbox) before opening a pull request. Any commit made on the *develop* branch (red square) will be merged to the *master* branch if the *develop* branch is stable overall (orange square).

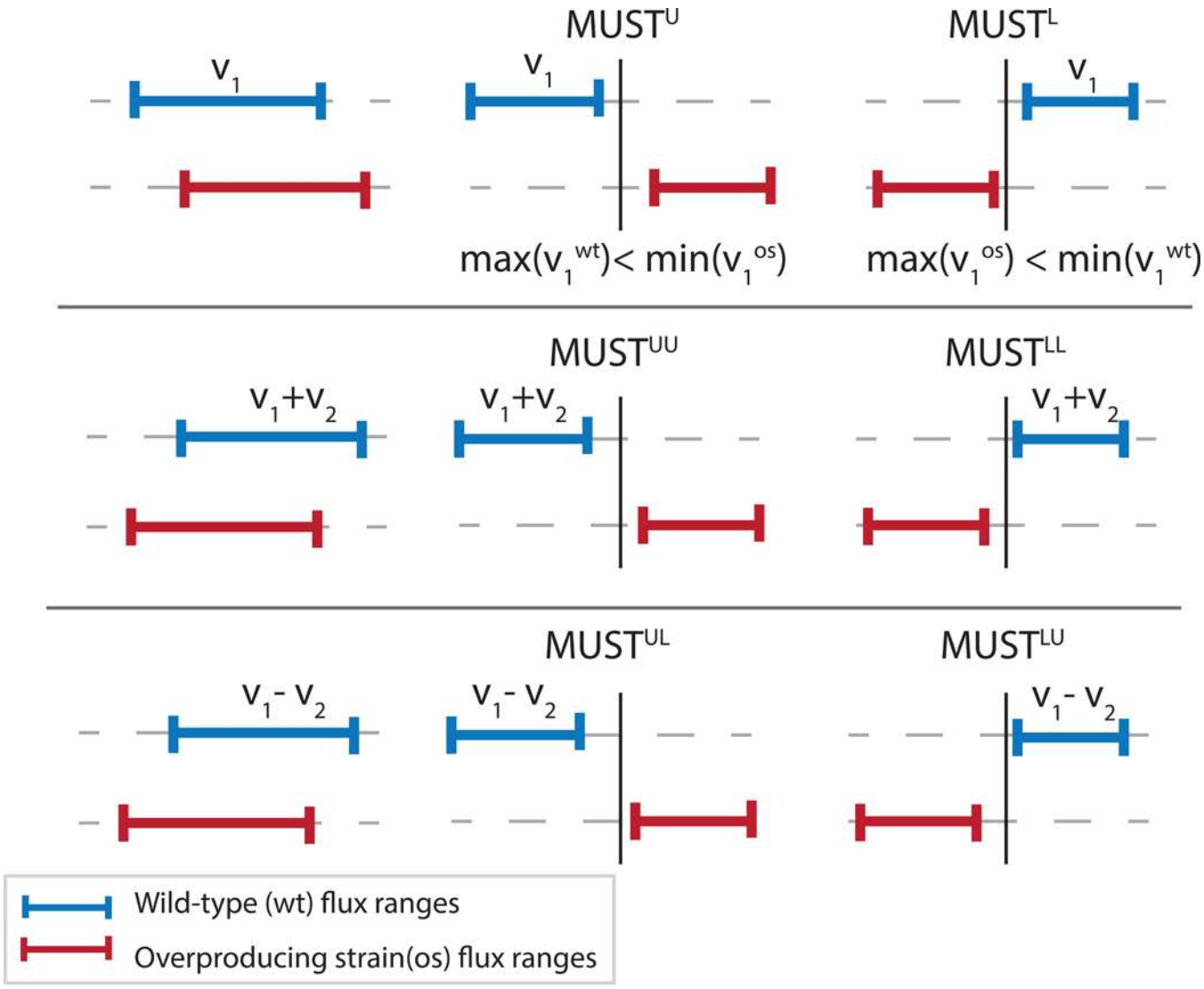

Figure 7:
Output of initialisation of the COBRA Toolbox with initCobraToolbox.

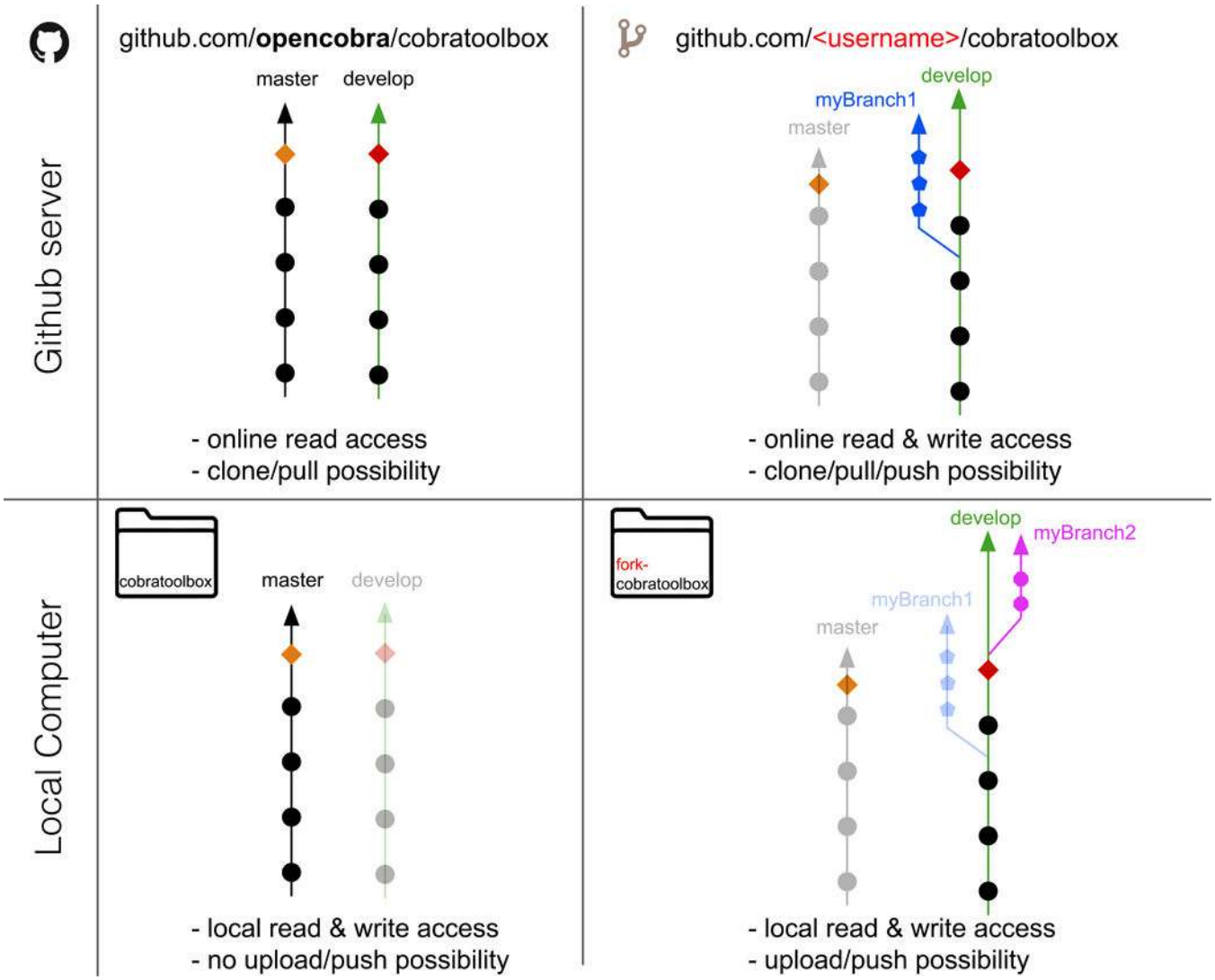

Figure 8: An energy generating stoichiometrically balanced cycle.

The smallest stoichiometrically balanced cycle that produces ATP at a maximal rate using the ATP synthase reaction, in Recon3D, with all internal reactions. All metabolite and reaction abbreviations are primary keys in the Virtual Metabolic Human database (https://vmh.uni.lu): reaction abbreviation, reaction name: ADK1m, adenylate kinase, mitochondrial; G5SDym, glutamate-5-semialdehyde dehydrogenase, mitochondrial; GLU5Km, glutamate 5-kinase, mitochondrial; P45027A15m, 5-beta-cytochrome P450, family 27, subfamily A, polypeptide 1; PPAm, inorganic diphosphatase; r0074, L-glutamate 5-semialdehyde:NAD+ oxidoreductase; HMR_3966, nucleoside-triphosphate giphosphatase; ATPS4mi, ATP synthase (four protons for one ATP); CYOR_u10mi, ubiquinol-6 cytochrome c reductase, Complex III; NADH2_u10mi, NADH dehydrogenase, mitochondrial; CYOOm2i, cytochrome c oxidase, mitochondrial complex IV.

```
 _____   _____   _____   _____     _____
/  ___| /  _  \ |  _  \ |  _  \   / ___ \    |   COnstraint-Based Reconstruction and Analysis
| |     | | | | | |_| | | |_| |  | |___| |   |   The COBRA Toolbox - 2017
| |     | | | | |  _  { |  _  /  |  ___  |   |
| |___  | |_| | | |_| | | | \ \  | |   | |   |   Documentation:
\_____| \_____/ |_____/ |_|  \_\ |_|   |_|   |   http://opencobra.github.io/cobratoolbox
                                              |

> Checking if git is installed ...  Done.
> Checking if the repository is tracked using git ...  Done.
> Checking if curl is installed ...  Done.
> Checking if remote can be reached ...  Done.
> Initializing and updating submodules ... Done.
> Adding all the files of The COBRA Toolbox ...  Done.
> Define CB map output... set to svg.
> Retrieving models ...   Done.
> TranslateSBML is installed and working properly.
> Configuring solver environment variables ...
- [*---] ILOG_CPLEX_PATH: /Users/syarra/Applications/IBM/ILOG/CPLEX_Studio1271/cplex/matlab/x86-64_osx
- [-*--] GUROBI_PATH: /Library/gurobi702/mac64/matlab
- [----] TOMLAB_PATH :  --> set this path manually after installing the solver ( see instructions )
- [----] MOSEK_PATH :  --> set this path manually after installing the solver ( see instructions )
Done.
> Checking available solvers and solver interfaces ... Done.
> Setting default solvers ... Done.
> Saving the MATLAB path ... Done.
- The MATLAB path was saved in the default location.

> Summary of available solvers and solver interface s

                 Support             LP     MILP      QP     MIQP     NLP
-------------------------------------------------------------------------
cplex_direct     full                 0        0       0        0       -
dqqMinos         full                 1        -       -        -       -
glpk             full                 1        1       -        -       -
gurobi           full                 1        1       1        1       -
ibm_cplex        full                 1        1       1        -       -
matlab           full                 1        -       -        -       1
mosek            full                 0        0       0        -       -
pdco             full                 1        -       1        -       -
quadMinos        full                 1        -       -        -       1
tomlab_cplex     full                 0        0       0        0       -
qpng             experimental         -        -       1        -       -
tomlab_snopt     experimental         -        -       -        -       0
gurobi_mex       legacy               0        0       0        0       -
lindo_old        legacy               0        -       -        -       -
lindo_legacy     legacy               0        -       -        -       -
lp_solve         legacy               1        -       -        -       -
opti             legacy               0        0       0        0       0
-------------------------------------------------------------------------
Total            -                    8        3       4        1       2

+ Legend: - = not applicable, 0 = solver not compatible or not installed, 1 = solver installed.


> You can solve LP problems using: 'dqqMinos' - 'glpk' - 'gurobi' - 'ibm_cplex' - 'matlab' - 'pdco' - 'quadMinos' - 'lp_solve'
> You can solve MILP problems using: 'glpk' - 'gurobi' - 'ibm_cplex'
> You can solve QP problems using: 'gurobi' - 'ibm_cplex' - 'pdco' - 'qpng'
> You can solve MIQP problems using: 'gurobi'
> You can solve NLP problems using: 'matlab' - 'quadMinos'

> Checking for available updates ...
> The COBRA Toolbox is up-to-date.
```

Figure 9:

The interventions predicted by the OptForce method for succinate overproduction in *E. coli* (AntCore model) under aerobic conditions. Reactions that need to be up-regulated (green arrows and labels) and knocked out (red arrows and labels) are shown in this simplified metabolic map. The strategies include up-regulation of reactions generating succinate such as isocitrate dehydrogenase (R2), α-ketoglutarate dehydrogenase or succinyl-CoA synthetase, along with knockout of reactions draining succinate such as succinate

dehydrogenase or fumarate hydratase. Note that each of these reactions may associate with one or more genes in *E. coli.*

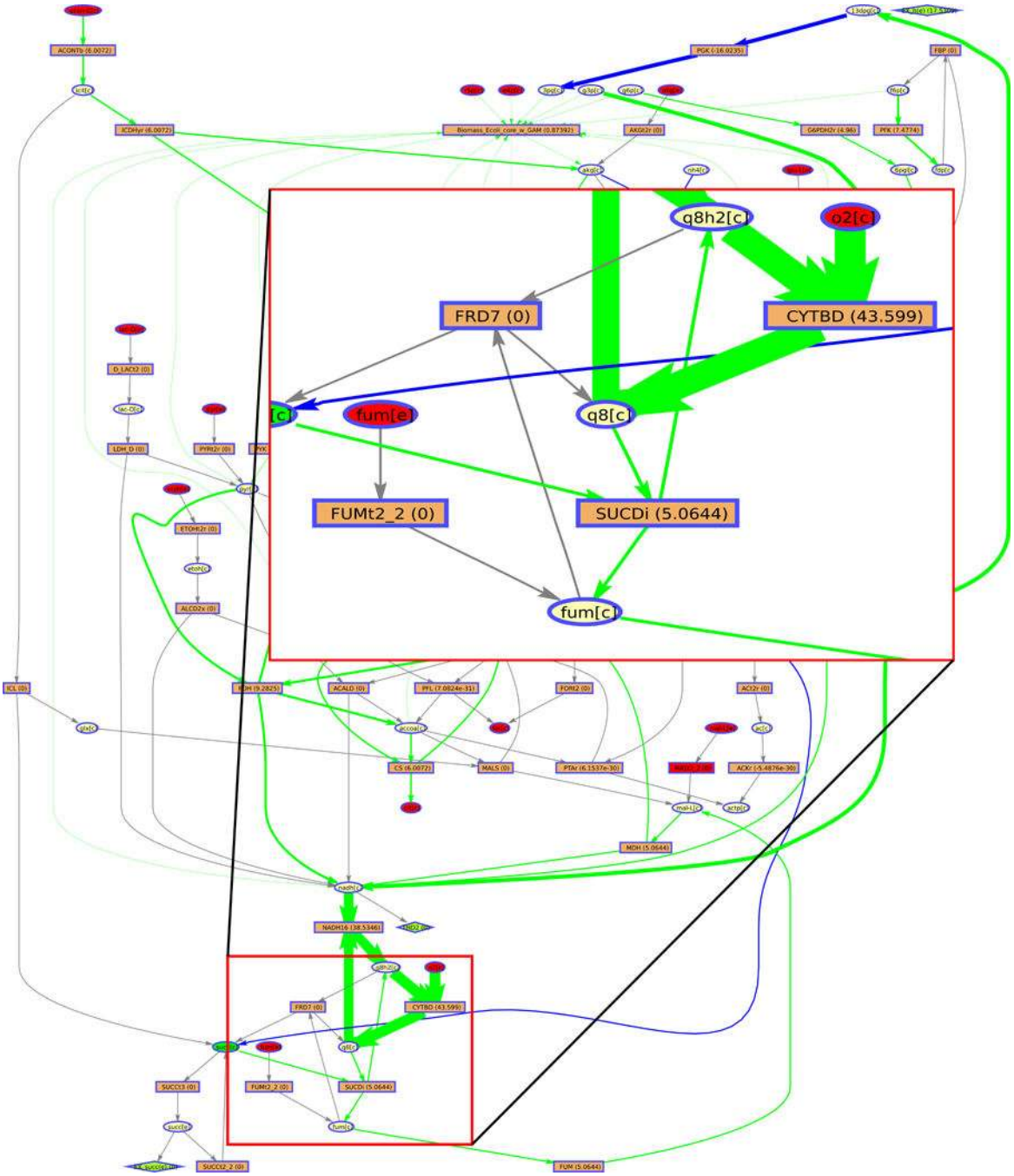

Figure 10: Qualitatively forward, quantitatively reverse reactions in a multi-compartmental, genome-scale model.

In Recon3D, the transformed reaction Gibbs energy could be estimated for 7, 215 reactions. Of these reactions, 2, 868 reactions were qualitatively assigned to be forward in the reconstruction, but were quantitatively assigned to be reversible using subcellular compartment specific thermodynamic parameters, the component contribution method, and broad bounds on metabolite concentrations (10^{-5} – 0.02 mol/L), except for certain cofactors. The geometric mean (green) and feasible range (between maximum and minimum) of estimated millimolar standard transformed reaction Gibbs energy ($A_r G'^m$, blue) and

transformed reaction Gibbs energy ($\Delta_r G'^m$, red) are illustrated. The relative uncertainty in metabolite concentrations versus uncertainty in thermochemical estimates is reflected by the relative breadth of the red and blue bars for each reaction, respectively. The reactions are rank ordered by the cumulative probability that millimolar standard transformed reaction Gibbs energy is less than zero, $P(\Delta_r G'^m < 0)$, (black descending line from left to right). This assumes that all metabolites are at a millimolar concentration *(1mM)* and a Gaussian error is assumed in component contribution estimates. In this ordering, forward transport reactions have $P(\Delta_r G'^m < 0) = 1$ (far left) and reverse transport reactions have $P(\Delta_r G'^m < 0) = 0$ (far right). In between, from left to right are biochemical reactions with decreasing cumulative probability of being forward in direction, subject to the stated assumptions. Alternative rankings are possible. The key point is to observe that the COBRA Toolbox is primed for quantitative integration of metabolomic data as the uncertainty in transformed reaction Gibbs energy associated with thermochemical estimates using the component contribution method is now significantly lower than the uncertainty associated with the assumption of broad concentration range.

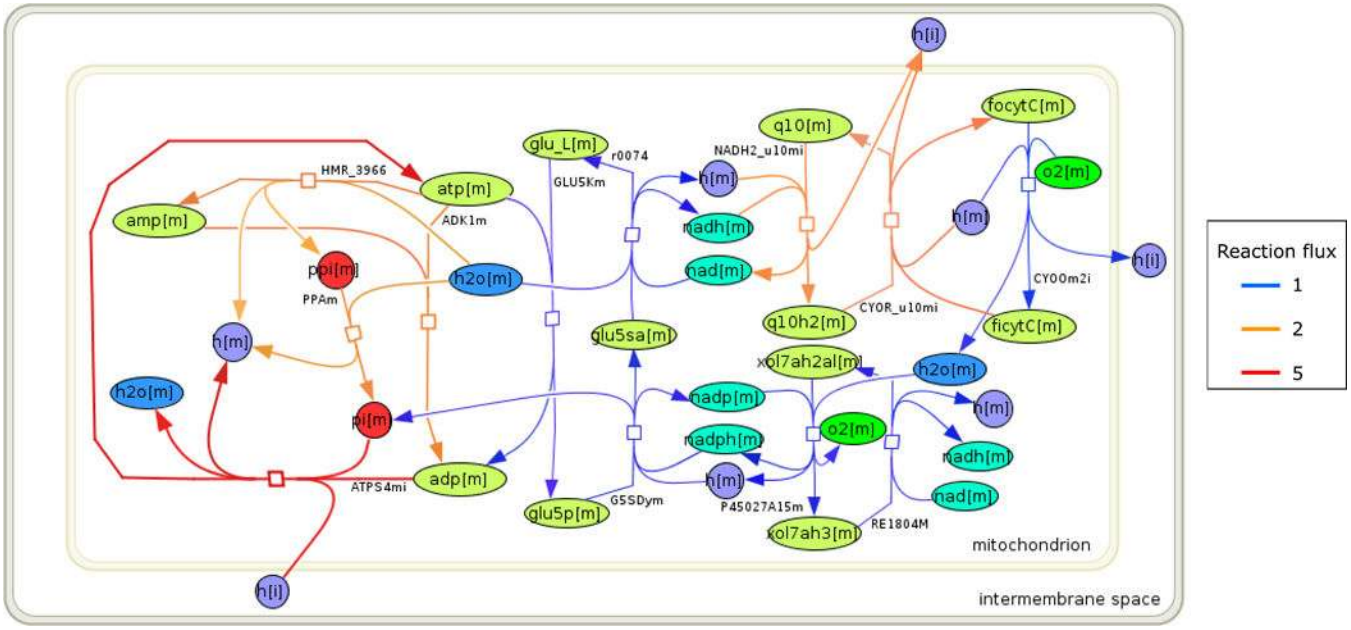

Figure 11:

Overlay of the flux vector for maximum ATP synthase flux, using flux balance analysis with regularisation of the flux vector. Active fluxes are highlighted (purple).

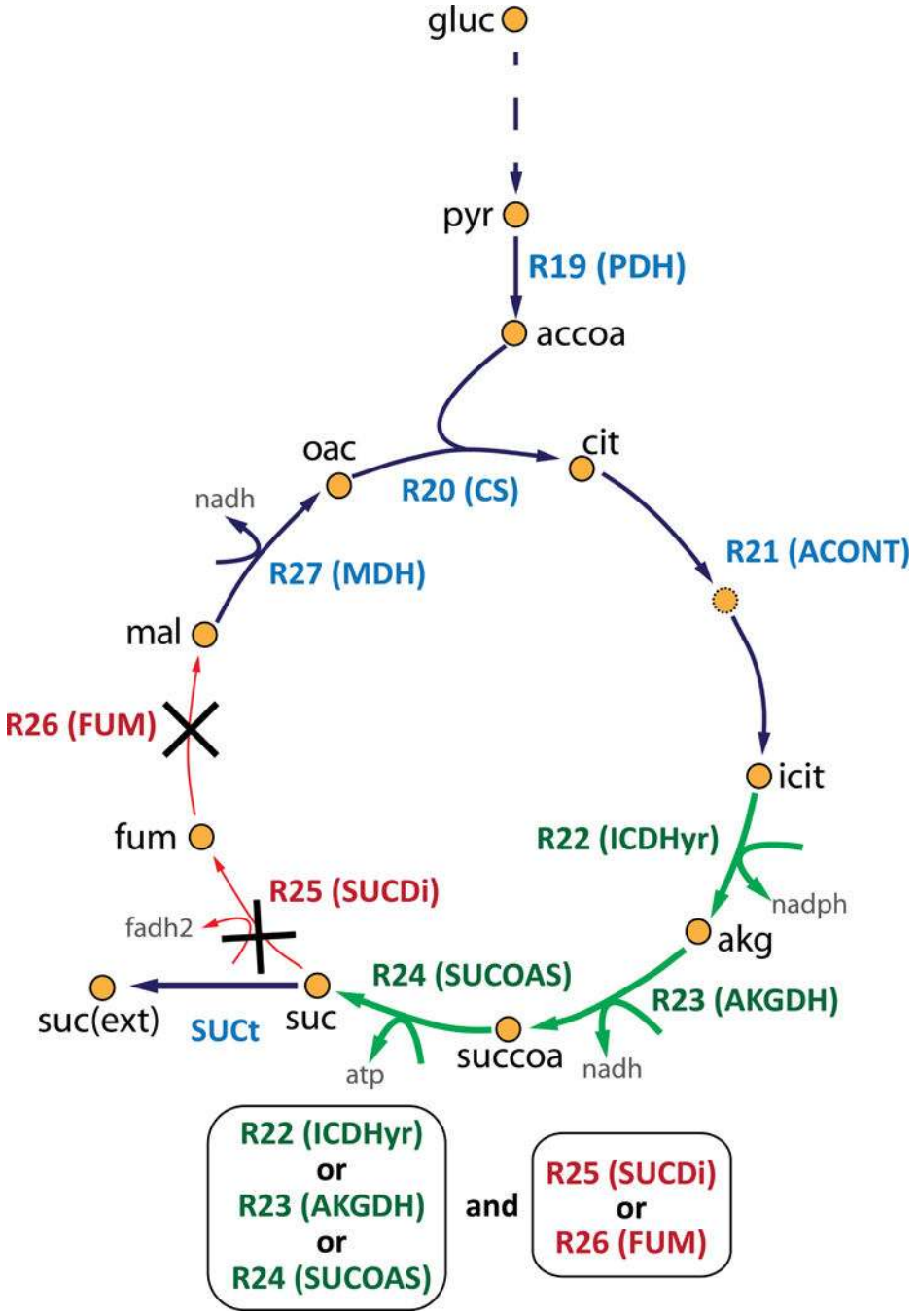

Figure 12:

Selective scope visualisation of the *E. coli* core model model by Paint4Net. Rectangles represent reactions with rates of fluxes in brackets; the red rectangles represent reactions with only one metabolite; ellipses represent metabolites; the red ellipses represent dead end metabolites; grey edges represent zero-rate fluxes; green edges represent positive-rate (forward) fluxes; and blue edges represent negative-rate (backward) fluxes. Network visualisation also enables zoom to specific regions.

Table 1:

Each method available in the COBRA Toolbox 3.0 is made accessible with a narrative tutorial that illustrates how the corresponding function(s) are combined to implement each COBRA method in the respective *src/* directories (https://github.com/opencobra/cobratoolbox/tree/master/src): *base* (**B**), *reconstruction* (**R**), *dataIntegration* (**I**), *analysis* (**A**), *design* (**D**), *visualisation* (**V**).

src/	Narrative	Novelty in the COBRA Toolbox 3.0 compared to 2.0
B	Initialise and verify the installation	Software dependency audit, e.g., solvers, binaries, git.
R	Input and output of reconstructions and models	Support for latest standards, e.g., SBML flux balance constraints64.
R	Reconstruction: rBioNet	New software for quality controlled reconstruction48.
R	Reconstruction: create a functional generic subnetwork	New methods for selecting different types of subnetworks.
R	Reconstruction exploration	New methods, e.g., find adjacent reactions.
R	Reconstruction refinement	Maintenance of internal model consistency, e.g., upon subnetwork generation29.
R	Numerical reconstruction properties	Flag a reconstruction requiring a multi-scale solver54.
R	Convert a reconstruction into a flux balance analysis model	Identification of a maximal flux and stoichiometrically consistent subset69.
I	Atomically resolve a metabolic reconstruction	New algorithms and methods for working with molecular structures, atom mapping, identification of conserved moieties110, 122.
I	Integration of metabolomic data	New methods for analysis of metabolomic data in a network context65, 142.
I	Integration of transcriptomic and proteomic data	New algorithms for generation of context-specific models89.
A	Flux balance analysis and its variants	New flux balance methods, multi-scale model rescaling and multi-scale solvers, additional solver interfaces, thermodynamically feasible methods42, 60, 128, 132, 143, 144.
A	Variation on reaction rate bounds in flux balance analysis	Increased computational efficiency.
A	Parsimonious flux balance analysis	New method for parsimonious flux balance analysis145.
A	Sparse flux balance analysis	New method for sparse flux balance analysis.
A	Gap filling	Increased computational efficiency82.
A	Adding biological constraints to a flux balance model	New methods for coupling reaction rates38, 146.
A	Testing biochemical fidelity	Human metabolic function test suite17.
A	Testing basic properties of a metabolic model (sanity checks)	New methods to minimise occurrence of modelling artefacts66.
A	Minimal spanning pathway vectors	New method for determining minimal spanning pathway vectors100.
A	Elementary modes and pathway vectors	Extended functionality by integration with CellNetAnalyzer55.
A	Minimal cut sets	Extended functionality by integration with CellNetAnalyzer147, 148, and new algorithms for genetic MCSs57.
A	Flux variability analysis	Increased computational efficiency101.
A	Uniform sampling of steady-state fluxes	New algorithm, guaranteed convergence to uniform distribution102.
A	Thermodynamically constrain reaction directionality	New algorithms and methods for estimation of thermochemical parameter estimation in multi-compartmental, genome-scale metabolic models126, 127.
A	Variational kinetic modelling	New algorithms and methods for genome-scale kinetic modelling68, 135–137.
D	Metabolic engineering and strain design	New methods, e.g., OptForce, interpretation of new strain designs. New modelling language interface to GAMS59.
V	Human metabolic network visualisation: ReconMap	New method for genome-scale metabolic network visualisation50, 51, 149.
V	Variable scope visualisation with automatic layout generation	New method for automatic visualisation of network parts141.

src/	Narrative	Novelty in the COBRA Toolbox 3.0 compared to 2.0
	Contributing to the COBRA Toolbox with MATLAB.devTools	New software application enabling contributions by those unfamiliar with version control software.
	Engaging with the COBRA Toolbox Forum	More than 800 posted questions with supportive replies connecting problems and solutions.

Table 2:

A selection of actively developed software applications with constraint-based modelling (COBRA) capabilities. GUI, graphical user interface. The COBRA Toolbox: https://opencobra.github.io/cobratoolbox, RAVEN: https://github.com/SysBioChalmers/RAVEN, CellNetAnalyzer: https://www2.mpi-magdeburg.mpg.de/projects/cna/cna.html, FBA-SimVis: https://immersive-analytics.infotech.monash.edu/fbasimvis, OptFlux: http://www.optflux.org, COBRA.jl: https://opencobra.github.io/COBRA.jl, Sybil: https://rdrr.io/cran/sybil, COBRApy: http://opencobra.github.io/cobrapy, CBMPy: http://cbmpy.sourceforge.net, SurreyFBA: http://sysbio.sbs.surrey.ac.uk/sfba, FASIMU: http://www.bioinformatics.org/fasimu, FAME: http://f-a-m-e.org, Pathway Tools: http://bioinformatics.ai.sri.com/ptools, KBase: https://kbase.us. The symbol † in the development column refers to an inactive project and the * to an active project. The column "Distrib." refers to the distribution channel. The label 'all' in the OS column means that the applications is compatible with Windows, Linux and macOS operating systems.

Name	Implementation	Interface	Development	Distrib.	OS
COBRA Toolbox	MATLAB (etc)	Script/Narrative	open source*	git	all
RAVEN150	MATLAB	Script	open source*	git	all
CellNetAnalyzer55	MATLAB (etc)	Script/GUI	closed source*	zip	all
FBA-SimVis151	Java + MATLAB	GUI	closed source†	zip	Windows
OptFlux152	Java	Script	open source*	svn	all
COBRA.jl42	Julia	Script/Narrative	open source*	git	all
Sybil53	R package	Script	open source*	zip	all
COBRApy40	Python	Script/Narrative	open source*	git	all
CBMPy52	Python	Script	open source*	zip	all
Scrumpy153	Python	Script	open source*	tar	all
SurreyFBA47	C++	Script/GUI	open source*	zip	all
FASIMU154	C	Script	open source†	zip	Linux
FAME155	Web-based	GUI	open source†	zip	all
PathwayTools43	Web-based	GUI/Script	closed source*	N/A	all
KBase41	Web-based	Script/Narrative	open source*	git	all

Table 3:

A description of the main fields of a standard model structure.

Field name	Size	Data Type	Field description
.b	$m \times 1$	double	The coefficients of the constraints of the metabolites ($S_v = b$).
.csense	$m \times 1$	char	The sense of the constraints represented by b, each row is either 'E' (equality), 'L' (less than) or 'G' (greater than).
.metCharges	$m \times 1$	numeric	The charge of the respective metabolite (NaN if unknown).
.metFormulas	$m \times 1$	cell of char	Elemental formula for each metabolite.
.metInChIString	$m \times 1$	cell of char	Formula for each metabolite in the InCHI strings format.
.metNames	$m \times 1$	cell of char	Full name of each corresponding metabolite.
.mets	$m \times 1$	cell of char	Identifiers of the metabolites.
.metSmiles	$m \times 1$	cell of char	Formula for each metabolite in SMILES Format.
.c	$n \times 1$	double	The objective coefficient of the reactions.
.grRules	$n \times 1$	cell of char	A string representation of the GPR rules defined in a readable format.
.lb	$n \times 1$	double	Lower bounds for fluxes through the reactions.
.rxnConfidenceScores	$n \times 1$	numeric	Confidence scores for reaction presence (0–5, with 5 being the highest confidence).
.rxnECNumbers	$n \times 1$	cell of char	E.C. number for each reaction.
.rxnNames	$n \times 1$	cell of char	Full name of each corresponding reaction.
.rxnNotes	$n \times 1$	cell of char	Description of each corresponding reaction.
.rxnReferences	$n \times 1$	cell of char	Description of references for each corresponding reaction.
.rxns	$n \times 1$	cell	Identifiers of the reactions.
.subSystems	$n \times 1$	cell of cell of char	subSystem assignments for each reaction.
.ub	$n \times 1$	double	Upper bounds for fluxes through the reactions.
.S	$m \times n$	numeric	The stoichiometric matrix containing the model structure (for large models a sparse format is suggested).
.geneNames	$g \times 1$	cell of char	Full name of each corresponding gene.
.genes	$g \times 1$	cell of char	Identifiers of the genes in the model.
.proteinNames	$g \times 1$	cell of char	Full name for each protein.
.proteins	$g \times 1$	cell of char	Proteins associated with each gene (one protein per gene).
.rxnGeneMat	$n \times g$	numeric or logical	Matrix with rows corresponding to reactions and columns corresponding to genes.
.compNames	$c \times 1$	cell of char	Descriptions of the Compartments (compNames(m) is associated with comps(m)).
.comps	$c \times 1$	cell of char	Symbols for compartments.
.osenseStr	1×3	char	The objective sense: either 'max' (maximisation) or 'min' (minimisation).

Table 4:

An overview of the types of optimisation problems solved by each optimisation solver. The interface to certain standard optimisation solvers is actively supported, whereas the interface to other non-standard solvers requires testing by the end user to ensure compatibility, while a legacy solver interface might require refinement before it becomes compatible with newer solver or MATLAB releases.

	Name	Version	Interface	LP	MILP	QP	MIQP	NLP
Active Support	DQQ	-	dqqMinos	⋆				
	GLPK	2.7+	glpk	⋆	⋆			
	GUROBI	7.0+	gurobi	⋆	⋆	⋆	⋆	
	ILOG CPLEX	12.7.1 +	ibm cplex	⋆	⋆	⋆		
	MATLAB	R2014b+	matlab	⋆				⋆
	MINOS	-	quadMinos	⋆				⋆
	MOSEK	8.0+	mosek	⋆	⋆	⋆		
	PDCO	-	pdco	⋆		⋆	⋆	⋆
	Tomlab CPLEX	8.0+	cplex_direct tomlab_cplex	⋆ ⋆	⋆ ⋆	⋆ ⋆	⋆	
Passive	OPTI	2.27+	opti	⋆	⋆	⋆	⋆	⋆
	QPNG	-	qpng			⋆		
	Tomlab SNOPT	8.0+	tomlab_snopt					⋆
Legacy	GUROBI	7.0+	Gurobi_mex	⋆	⋆	⋆	⋆	
	LINDO	2.0+	lindo_old	⋆				
		R2014b+	lindo_legacy	⋆				
	MATLAB		lp_solve	⋆				
				⋆				