Fluxes of life: bioinformatics for metabolic flux quantification in isotopic non-steady-state

Fluxomics matters

Life is a continuous process of adaptation to new environmental conditions and constantly asking for the right adjustments that ensure the survival of a life form. A very suitable reflection of such dynamic changes can be found in the metabolism of all living organisms.

Metabolism describes the entirety of all present biochemical reactions and from microorganism to higher life forms qualitatively much is known. However, there is a huge lack of knowledge with respect to the quantitative metabolic parameters which are essential to understand complex metabolic systems under all conditions. This absence of parameters is a huge obstacle for modeling and understanding metabolism in humans, animals and organisms in its entirety. Hence, this thesis and the four years of PhD research were devoted to fluxomics which reveals the dynamic flow of mass between metabolites and aims to close these knowledge gaps.

Fluxomics studies are quite complex and suffer from several bottlenecks such as non-intuitive mathematical models or difficult experimental situations. This thesis addressed some of these impediments which are discussed in the following. Finally, an outlook to future developments in fluxomics and their possible application in medical diagnosis are given.

Experimentalists model in ‘sloppy’ parameter space

Occasionally, there can be a large discrepancy between modelers and experimentalists with respect to knowledge about mathematical and computational models. In par-
Summary

In particular, computational models aiming to simulate isotope distributions and metabolic fluxes are complex constructs, a fact which may even make experimentalists hesitate to take the effort of analyzing their data in a sophisticated way. To bridge this gap and establish a more intuitive way to build and simulate metabolic models the two computer programs FluxSimulator and FluxSimulator$_{CPN}$ were developed and described in Chapter 2 and 3 of this thesis.

Common implementations of metabolic models use ordinary differential equations (ODEs) to simulate the time dependent metabolic dynamics. Although ODEs are an appropriate tool for such simulations the model implementation in computer readable format is error prone, inflexible and not practicable for unexperienced people. Therefore, FluxSimulator (Chapter 2) introduces a user-friendly specification scheme of the metabolic pathways via three plain text files. Afterwards, the entire system of ODEs is assembled and simulated automatically.

The flexible model specification of FluxSimulator was used to simulated the isotope distribution in a metabolic model. Afterwards, the results were compared to a hard-coded implementation of the same model using the modeling environment Berkeley Madonna. Both simulations showed an identical dynamic behavior. However, in contrast to Berkeley Madonna, FluxSimulator is suitable for a broader audience including experimental practitioners with possibly less mathematical background and experience.

Although FluxSimulator is already a good step forward to create ‘modeling experimentalists’ this approach is maybe still not attractive as it still is not in line with their intuition. Therefore, FluxSimulator$_{CPN}$ (Chapter 3) uses a more graphical approach implemented via Petri nets. Petri nets are close in structure and functionning to the metabolic models they represent and accurate enough for many biological simulation purposes.

A metabolic test model and a model of the tricarboxylic acid (TCA) cycle were simulated both with the discrete and the continuous colored Petri net representation implemented in FluxSimulator$_{CPN}$. In addition, both models were simulated with FluxSimulator as a reference. All simulations revealed a consistent behavior among all approaches. In the case of continuous Petri nets this is not a surprise as they may be seen as graphical ODE representations with simulations resembling an integration of ordinary differential equations by the Euler method. However, discrete colored Petri nets enable to deal with ‘real’ molecules in the form of tokens, making this approach less abstract and more intuitive for experimentalists compared to ODE approaches.

Both introduced simulation programs (FluxSimulator and FluxSimulator$_{CPN}$) offer a flexible and intuitive way to implement and simulate isotope distributions in metabolic models. Therefore, they are perfectly suited to determine parameter combinations with either a large (‘stiff’ parameter direction) or very small (‘sloppy’ parameter direction) effect on model predictions when changing the parameters in a correlated way. By simulating ensembles of parameter combinations it is hence possible to decide which parameters can be estimated precisely and imprecisely.
Flux quantification under challenging conditions

Flux quantification is challenging and the quality of the quantified flux values heavily depends on the experimental data available. In recent years, many fluxomics approaches were developed for experimental protocols designed for metabolic and isotopic steady-state, i.e. the metabolite concentrations and the isotope compositions of the metabolites do not change over time. Unfortunately, steady-state approaches require long experiments and therefore a lot of isotopic labeled substrate which makes experiments expensive. In addition, steady-state experiments are not always feasible in mammalian systems where such states are usually brief. Yet another obstacle frequently arising in mammalian models is the frequent impossibility of obtaining time series measurements.

To enable flux quantification in the challenging situation of isotopic non-steady-state without available time series data the flux simulation and estimation program FluxEs was developed (Chapter 4). In particular, FluxEs makes flux estimation possible in single tissue samples in isotopic non-steady-state. FluxEs was tested by quantitating aerobic metabolic fluxes, like the tricarboxylic acid (TCA) cycle flux, from NMR multiplets measured from pig heart samples taken in vivo. The pigs were divided into a control group and a group with induced cardiac stress. As the oxygen consumption is coupled to the TCA cycle the flux quantifications were compared to the myocardial oxygen consumption quantified from blood gas and blood flow measurements.

The flux quantification using FluxEs revealed the expected individual differences in energy metabolism among pigs, even under the same experimental conditions. In addition, spatial variation in metabolism could be observed in each single heart, which had previously been found at the level of TCA cycle enzyme activities and blood supply of oxygen. Oxygen consumptions calculated from the metabolic fluxes and measured from blood gas correlated well and the higher workload in the cardiac stress group was clearly reflected by both an increase of the TCA cycle flux and higher oxygen consumption.

FluxEs offers like FluxSimulator an intuitive input format for assembling the mathematical model representation. This makes FluxEs applicable to a broad range of metabolic systems and the automatic flux quantification convenient for life scientists without experience in computer programming.

The unique capabilities of FluxEs are demonstrated in an application study on pig hearts exhibiting a septic shock-like state, a model resembling human septic shock with hypotension (Chapter 5). Computational analysis using FluxEs was able to reveal a mismatch of regional left ventricular oxygen delivery to demand, associated with decreasing global function. The increase in oxygen costs for excitation-contraction coupling may both contribute to the decrease in global function and help to maintain global coronary blood flow caused by global metabolic autoregulation. This enabled us to draw conclusions about the causes and effects of reversible systolic myocardial dysfunction which is a characteristic feature of human septic shock.
Flux quantification under more challenging conditions

Experiments in metabolic steady-state are not always feasible and the changing metabolite concentrations pose a big problem for the computational quantification of metabolic fluxes. Quantification of metabolic fluxes might be successful if the changes of the internal metabolite concentrations are known and time course data of label incorporation in multiple key metabolites is measured. However, if such complete datasets are missing reliable flux estimation is a great challenge.

Chapter 6 describes a flux analysis approach using a complex ODE model of the metabolism of human faecal microbiota. The experimental data under metabolic and isotopic non-steady-state conditions were measured using an in vitro model of the human proximal colon (TIM-2).

The analysis made it possible to determine the relative flux distribution of four major pathways in the metabolic model. This demonstrates that flux determination is possible even in situations with poor knowledge about dynamic changes in natural metabolite concentration. The experimental design and the analysis of the results enables to determine time-resolved effects of nutrition on the flux distribution in human faecal microbiota. Nevertheless, design principles for nonstationary $^{13}$C experiments as proposed by Nöh and Wiechert are needed if parameter estimation and accuracy should be improved in the future.

Future directions of fluxomics

To analyze complex metabolic systems, it is most useful if one can divide large networks into modules that can be isolated conceptually and experimentally to be studied independently in detail. Models such as the carbon transition networks given in this thesis are usually part of a bigger system. If the fluxes in the pathway dominate strongly over those entering and leaving via side paths, then it is possible to study this model as a module. It is probably difficult to divide the metabolic system completely into modules that can be studied independently, because metabolic pathways overlap and interact extensively. However, if it is possible to study a certain part in isolation, this makes it possible to define the function of that part of the system accurately. Connecting the modules again to form the whole system would be the ideal strategy to study a large system.

Given that there is so much information available on the connectivity of metabolism, the question may be posed whether it is time to start building quantitative and dynamic models of human and animal metabolism. Gradually, by trial and error, building a more comprehensive model of the human metabolic system may help to integrate and understand the enormous amount of experimental information on human and animal metabolism. Building a valid model will be possible only by making flux predictions on new experiments and correcting the model if the predictions prove wrong. In this way, dynamic models of metabolism, containing many pathways and metabolites, may prove their value to integrate the extensive knowledge on thousands of metabolites. This becomes even more desirable when analyzing and
predicting metabolic system behavior during a disease process or therapeutic intervention. Because it is inevitable to face the challenge of developing models of human and animal metabolism, there is a need to deal with the large scale of the system and in particular with the substantial number of imprecisely known kinetic parameters.

One possibility to tackle imprecisely known parameters is the application of ensemble simulations to explore the range of plausible parameter values. To this end, many simulations are done with different parameter sets that cover the plausible part of multidimensional parameter space, taking correlation between the parameters into account. Importantly, such simulations can also be used to explore the effect of measurement noise on the confidence regions of parameters estimated from experimental data.

Yet another possibility to improve the knowledge about kinetic parameters would be the application of multiscale analysis. This means that measurements at various aggregation levels are taken into account. For instance, not only the properties of enzymes or isolated mitochondria could be incorporated in an analysis, but also the measured response of a whole pathway or network of connected enzymes and organelles in the cell. Experimentally, this may be accomplished by measuring the time course of adaptation to altered cellular workloads, such as increased muscle contraction frequency, neural firing rate or secretion of hormone. A further example is the measurement of metabolic fluxes in specific pathways under various steady-state conditions in relation to the metabolite levels in the pathway.

Care must be taken that the model of the metabolic pathway and carbon distribution routes is compatible with the studied organ and cell type. The model for the cardiac study used in Chapter 4 and 5 was in particular designed and tested for heart muscle in vivo. Although the metabolic pathways of the TCA cycle and related amino acids incorporated in this model are almost universally found in other organs and cell types, the activity of various anaplerotic pathways varies under experimental conditions. Therefore, models of metabolic pathways and carbon distributions must be adapted to accurately reflect the precise cell type under study. It is desirable that the model’s suitability to accurately quantitate metabolic fluxes is examined on an organ by organ and cell-type by cell-type basis.

A crucial aspect is organization of the modeling process in such a way that, if inevitable mistakes are made in early model versions, the interplay between computer simulations and experimental tests results in a gradual improvement of the model. We must aim to make the modeling process the driving force behind metabolic experimentation and data collection, such that it becomes the vehicle for integration of knowledge and understanding of the complete metabolic system.

**Fluxomics in drug discovery**

The rate of energy production in the heart reflects its health and ability to pump blood. Energy production is proportional to local oxygen consumption which is coupled to aerobic metabolism of fatty acids or carbohydrates. If blood flow and hence oxygen delivery to heart tissue is reduced during coronary heart disease, the pump
capacity of the heart is threatened and Ischemic Heart Disease (IHD) evolves.

IHD is the leading single cause of death in the western world. In fact, the estimated number of patients suffering from stable angina aggregate to 16.5 million in US alone. Due to the prevalence of IHD and its associated morbidity and mortality there is a high need for new effective medicines. This is also reflected by the hundreds of clinical trials in progress to develop such pharmaceutical interventions. However, high failure rates of potential drugs in clinical trials and associated costs are a major obstacle in the drug development process. Hence, the ability to detect ineffective drug candidates at an early stage of the drug discovery and development process is of potential value for any pharmaceutical company.

During IHD usually small areas in the heart are affected, much more than the rest of the heart. In those hard hit areas cells may die, and arrhythmias and chest pain may be caused. One key therapeutic goal is to maintain metabolism and energy production in these focal areas despite the strong reduction of blood flow. To reach this goal it is essential that energy metabolism and usage of fatty acid and carbohydrates in these small areas of cardiac tissue can be measured during drug development.

In order to predict the efficacy of potential drug candidates one has to quantify the energy turnover in the ischemic regions of the heart because it is exactly this process (and not the cellular levels of ATP for instance) that is affected the earliest in hypoxic tissue. The experimental and analytical approach described in Chapter 4 of this thesis enable to quantify metabolic fluxes and energy turnover. This offers a unique opportunity to see whether candidate drugs increase energy metabolism and therefore survival and function of heart cells threatened by ischemia.

Conclusions
Quantitative knowledge about metabolic parameters is indispensable for modeling and understanding metabolism in living organisms in its entirety. Hence, this PhD research was devoted to fluxomics methods narrowing the knowledge gap about such quantitative metabolic parameters.

Two programs to simulate $^{13}$C tracer experiments (FluxSimulator (Chapter 2) and FluxSimulator$^{CPN}$ (Chapter 3)) were developed and successfully tested. Both programs offer a different, but flexible and intuitive way to implement and simulate carbon isotope experiments. This makes the complex world of modeling more accessible to experimentalists who have great knowledge about biology but less experience in complex mathematical models. In this way, more biologically reliable models can be developed.

However, computer programs that enable to simulate $^{13}$C tracer experiments using educated guesses about metabolic parameters are only a first step and a computer program able to quantitate metabolic parameters from experimental data is highly desirable. In addition, such a computer program should be both easy to use and able to quantify metabolic parameters under challenging conditions like single time point measurements in metabolic steady but isotopic non-steady-state. For this purpose, the computer program FluxEs was developed and successfully tested by quantitating
aerobic metabolic fluxes from pig heart samples (Chapter 4). FluxEs was subsequently used to analyze data from pig hearts studied under stun conditions. As an example, a chapter is included for a study on a septic shock-like state (Chapter 5). Such hearts are a model resembling human septic shock with hypotension and the results revealed a decreasing global heart function connected to a mismatch of regional left ventricular oxygen delivery to demand. Without the unique capabilities to quantitate metabolic fluxes from single tissue samples in isotopic non-steady-state such observations would not be possible.

The quantification of metabolic parameters on experimental data gathered in metabolic steady but isotopic non-steady-state is already very challenging. However, things get even more complex when moving to metabolic transient conditions. Chapter 6 of this thesis describes an approach to quantitate metabolic parameters in human faecal microbiota in this challenging situation and shows that conclusions about metabolic flux distributions are possible.

In conclusion, during this PhD research new fluxomics methods to quantitate metabolic parameters were investigated and developed. These methods provide a solid base for future developments directed to quantify metabolic parameters and help to better understand the metabolism of living organisms. Consequently, fluxomics may become an important tool for decision making in complex application areas like drug development.