A New Approach to Flux Coupling Analysis of Metabolic Networks*

Abdelhalim Larhlimi and Alexander Bockmayr

FB Mathematik und Informatik, Freie Universität Berlin,
Arnimallee, 3, 14195 Berlin, Germany
e-mail: larhlimi@mi.fu-berlin.de,bockmayr@mi.fu-berlin.de

Abstract. Flux coupling analysis is a method to identify blocked and coupled reactions in a metabolic network at steady state. We present a new approach to flux coupling analysis, which uses a minimum set of generators of the steady state flux cone. Our method does not require to reconfigure the network by splitting reversible reactions into forward and backward reactions. By distinguishing different types of reactions (irreversible, pseudo-irreversible, fully reversible), we show that reaction coupling relationships can only hold between certain reaction types. Based on this mathematical analysis, we propose a new algorithm for flux coupling analysis.

1 Introduction

Constraint-based methods for network-based metabolic pathway analysis have attracted growing interest in recent years [6]. The constraints that have to hold in a metabolic network at steady state include stoichiometry and thermodynamic irreversibility. These two classes of constraints not only determine all the possible flux distributions over the metabolic network at steady state. They also induce different dependencies between the reactions. For example, some reactions, which are called *blocked reactions* [1] or *strictly detailed balanced reactions* [9], may be incapable of carrying flux under steady state conditions. Another possibility are *coupled reactions* [1], where a zero flux through one reaction implies a zero flux through other reactions. Since a zero flux through some reaction corresponds to the deletion of the corresponding gene, such dependencies also link the metabolic to the gene regulatory network.

The elucidation of blocked and coupled reactions helps to better understand metabolic interactions within cellular networks. In this context, the *Flux Coupling Finder (FCF)* framework [1] has been developed to identify blocked and coupled reactions in metabolic networks. Maximizing the flux through a reaction under stoichiometric and thermodynamic constraints allows one to decide whether this reaction is blocked. Similarly, comparing flux ratios is the basis to determine whether two reactions are coupled.

* Supported by the DFG Research Center MATHEON "Mathematics for key technologies"

The FCF framework [1] requires a reconfiguration of the metabolic network. All reversible reactions are split into a forward and a backward reaction, which both are constrained to be non-negative. This implies that the number of variables and constraints increases. Since FCF uses linear fractional programming to identify the maximum and minimum flux ratios for every pair of metabolic fluxes, a very big number of linear optimization problems has to be solved. Therefore, FCF may not scale well for genome-scale models of complex microorganisms which involve a large number of reactions. Furthermore, as a consequence of the network reconfiguration, FCF cannot compute directly coupling relationships for reversible reactions. Since all reversible reactions are split into a forward and a backward reaction, FCF only computes interactions between reaction directions (see Fig. 1). A post-processing step is needed to deduce reaction couplings involving reversible reactions in the original network.

The goal of this work is to develop a new method to identify blocked and coupled reactions in a metabolic network at steady state. The organization of the paper is as follows. We start in Sect. 2 with some basic facts from polyhedral theory and the definition of the steady state flux cone of a metabolic network. This leads to a classification of reactions according to their reversibility type. In Sect. 3, we show mathematically that coupling relationships depend on the reversibility type of the reactions. Our main result is that reaction couplings can only hold between certain reaction types and that they can be computed using a minimum set of generating vectors of the flux cone. In Sect. 4, we propose a new algorithm to identify blocked and coupled reactions. Finally, some computational results are given to compare the new method with the original FCF framework.

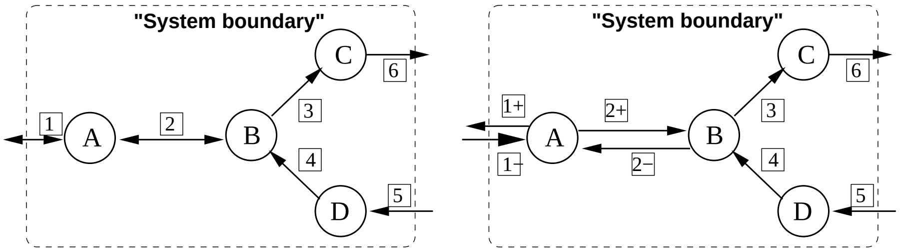
2 The steady state flux cone of a metabolic network

2.1 Polyhedral cones

We start with some basic facts about polyhedral cones (see e.g. [8]). A set $C \subseteq \mathbb{R}^n$ is called a *(convex) cone* if $\alpha x + \beta y \in C$, whenever $x, y \in C$ and $\alpha, \beta \geq 0$. A cone C is *polyhedral*, if $C = \{x \in \mathbb{R}^n \mid Ax \geq 0\}$, for some real matrix $A \in \mathbb{R}^{m \times n}$. In this case, $\text{lin.space}(C) = \{x \in \mathbb{R}^n \mid Ax = 0\}$ is called the *lineality space* of C. A cone C is *finitely generated* if there exist $x^1, \ldots, x^s \in \mathbb{R}^n$ such that $C = \text{cone}\{x^1, \ldots, x^s\} \stackrel{\text{def}}{=} \{\alpha_1 x^1 + \ldots + \alpha_s x^s \mid \alpha_1, \ldots, \alpha_s \geq 0\}$. A fundamental theorem of Farkas-Minkowski-Weyl asserts that a convex cone is polyhedral if and only if it is finitely generated. In the sequel, we will consider only polyhedral cones.

An inequality $a^T x \geq 0, a \in \mathbb{R}^n \setminus \{0\}$, is *valid* for C if $C \subseteq \{x \in \mathbb{R}^n \mid a^T x \geq 0\}$. The set $F = C \cap \{x \in \mathbb{R}^n \mid a^T x = 0\}$ is then called a *face* of C. Let t be the dimension of the lineality space of C. A *minimal proper face* of C is a face of dimension $t + 1$.

If we select for each minimal proper face $G^k, k = 1, \ldots, s$, a vector $g^k \in G^k \setminus \text{lin.space}(C)$, and a vector basis $b^1, \ldots, b^t$ of $\text{lin.space}(C)$, then for all $v \in C$,



(a) **Network example** (b) **Reconfigured network**

Fig. 1. The reversible reaction 2 in Fig. 1(a) is split into a forward and backward reaction 2^+ and 2^-. According to FCF, a zero flux through reaction 3 (resp. 4) implies a zero flux through reaction 2^+ (resp. 2^-), i.e., a negative (resp. positive) flux through the reversible reaction 2. However, neither a zero flux through reaction 3 nor a zero flux through reaction 4 implies a zero flux through reaction 2.

there exist $\alpha_k, \beta_l \in \mathbb{R}, \alpha_k \geq 0$ such that

$$v = \sum_{k=1}^{s} \alpha_k g^k + \sum_{l=1}^{t} \beta_l b^l. \tag{1}$$

2.2 The steady state flux cone

Mathematically, the stoichiometric and thermodynamic constraints that have to hold in a metabolic network have the form [5]

$$Sv = 0,\ v_i \geq 0,\ \text{for } i \in \mathit{Irr}. \tag{2}$$

Here, S is the $m \times n$ stoichiometric matrix of the network, with m metabolites (rows) and n reactions (columns), and $v \in \mathbb{R}^n$ is the *flux vector*. The set $\mathit{Irr} \subseteq \{1, \ldots, n\}$ defines the *irreversible* reactions in the network, while $\mathit{Rev} = \{1, \ldots, n\} \setminus \mathit{Irr}$ is the set of *reversible* reactions. The set of all solutions of the constraint system (2), which corresponds to the set of all possible flux distributions through the network at steady state, defines a polyhedral cone,

$$C = \{v \in \mathbb{R}^n \mid Sv = 0,\ v_i \geq 0, i \in \mathit{Irr}\} \tag{3}$$

which is called the *steady state flux cone*.

Example 1. Consider the hypothetical network ILLUSNET depicted in Fig. 2. It consists of thirteen metabolites $(A, \ldots, O)$, and nineteen reactions $(1, \ldots, 19)$. The steady state flux cone is defined by $C = \{v \in \mathbb{R}^{19} \mid Sv = 0, v_i \geq 0 \text{ for all } i \in Irr\}$, with the stoichiometric matrix S and the set of irreversible reactions $\mathit{Irr} =$

$\{1, 2, 3, 4, 5, 6, 7, 8\}$. Fig. 2 shows four pathways

$$\begin{aligned}
g^1 &= (2, 2, 1, 0, 0, 0, 0, 0, \quad 2, \quad 2, \quad 1, \quad 1, 0, 0, 0, 0, 0, 0, 0),\\
g^2 &= (0, 0, 1, 2, 0, 0, 0, 0, \quad 0, \quad 0, -1, -1, 2, 0, 0, 0, 0, 0, 0),\\
g^3 &= (0, 0, 0, 0, 1, 1, 1, 0, -1, -1, \quad 0, \quad 0, 0, 0, 0, 0, 0, 0, 0),\\
g^4 &= (0, 0, 0, 0, 1, 1, 0, 1, -1, -1, \quad 0, \quad 0, 0, 0, 1, 1, 0, 0, 0).
\end{aligned}$$

representing the four minimal proper faces $G^k, k = 1, 2, 3, 4$ of the network. The lineality space $\text{lin.space}(C) = \{v \in C \mid v_i = 0, i \in \mathit{Irr}\}$ has dimension 2. It can be generated by the pathways

$$\begin{aligned}
b^1 &= (0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, -1, 1, 1, 1, 0, 0, 0),\\
b^2 &= (0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, -1, 1, 0, 0, 1, 1, 0).
\end{aligned}$$

An arbitrary pathway $v \in C$ can be written in the form combination $v = \sum_{k=1}^{4} \alpha_k g^k + \sum_{l=1}^{2} \beta_l b^l$, for some $\alpha_k \geq 0$ and $\beta_1, \beta_2 \in \mathbb{R}$.

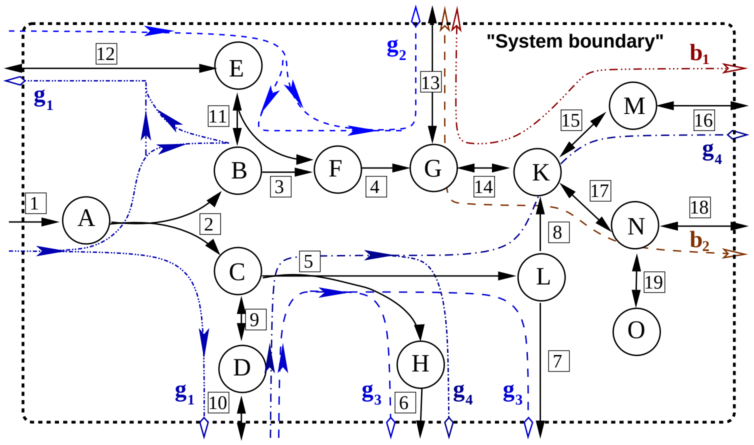


Fig. 2. Network example (ILLUSNET) with representative pathways

2.3 Reaction classification

A reversible reaction $j \in \mathit{Rev}$ is called *pseudo-irreversible* [4] if $v_j = 0$, for all $v \in \text{lin.space}(C)$. A reversible reaction that is not pseudo-irreversible is called *fully reversible.*

Inside each minimal proper face, the irreversible and the pseudo-irreversible reactions take a unique direction. More precisely, we have the following properties.

Theorem 1 ([4]). *Let G be a minimal proper face of the flux cone C and let $j \in \{1, \dots, n\}$ be a reaction.*

- *If $j \in Irr$ is irreversible, then $v_j > 0$, for all $v \in G \setminus \text{lin.space}(C)$, or $v_j = 0$, for all $v \in G$. Furthermore, $v_j = 0$, for all $v \in \text{lin.space}(C)$.*
- *If $j \in Rev$ is pseudo-irreversible, then the flux v_j through j has a unique sign in $G \setminus \text{lin.space}(C)$, i.e., either $v_j > 0$, for all $v \in G \setminus \text{lin.space}(C)$, or $v_j = 0$, for all $v \in G \setminus \text{lin.space}(C)$, or $v_j < 0$, for all $v \in G \setminus \text{lin.space}(C)$. For all $v \in \text{lin.space}(C)$, we have again $v_j = 0$.*
- *If $j \in Rev$ is fully reversible, there exists $v \in \text{lin.space}(C)$ such that $v_j \neq 0$. We can then find pathways $v^+, v^-, v^0 \in G \setminus \text{lin.space}(C)$ with $v_j^+ > 0$, $v_j^- < 0$ and $v_j^0 = 0$.*

Example 2. In the ILLUSNET network, the reactions $9, 10, 11$ and 12 are pseudo-irreversible, while reactions $15, 16, 17, 18$ are fully reversible. In the context of the minimal proper face G^1, all the pseudo-irreversible reactions become positive, i.e., $v_i > 0$ for all $v \in G^1 \setminus \text{lin.space}(C)$, while the pseudo-irreversible reactions 9 and 10 become negative in the context of the faces G^3 and G^4. The reaction 19 has zero flux in all the minimal proper faces and in the lineality space of the flux cone.

3 Flux coupling analysis based on the reversibility type of reactions

For the rest of the paper, we assume that $G^k, k = 1, \dots, s$, are the minimal proper faces of the steady state flux cone C, represented by vectors $g^k \in G^k \setminus \text{lin.space}(C)$, and that $b^l, l = 1, \dots, t$, is a vector basis of $\text{lin.space}(C)$. Based on Theor. 1, we define the following decomposition of the reaction set $\{1, \dots, n\}$, which reflects that pseudo-irreversible reactions taking the same direction in all minimal proper faces behave like irreversible reactions.

- $Irev = Irr \cup \{i \mid i \text{ is pseudo-irreversible and } v_i \geq 0, \text{ for all } v \in C \text{ or } v_i \leq 0, \text{ for all } v \in C\}$.
- $Prev = \{i \mid i \text{ is pseudo-irreversible and there exist } v^+, v^- \in C \text{ such that } v_i^+ > 0, v_i^- < 0\}$,
- $Frev = \{i \mid i \text{ is fully reversible}\}$.

Definition 1. *A reaction $i \in \{1, \dots, n\}$ is* blocked *if $v_i = 0$, for all $v \in C$. Otherwise, the reaction i is* unblocked.

First, we characterize blocked reactions using generators of the cone. The following proposition follows directly from (1).

Proposition 1. *For any reaction $i \in \{1, \dots, n\}$, the following are equivalent:*

1. *The reaction i is blocked.*
2. *$g_i^k = 0$, for all $k = 1, \dots, s$, and $b_i^l = 0$, for all $l = 1, \dots, t$.*

In our analysis, we will first compute the blocked reactions using Prop. 1. Afterwards, we will identify coupled reactions based on the subsequent results. Here, it is enough to consider only unblocked reactions. The proofs of these properties can be found in Appendix A.

Definition 2 ([1]). *Let i, j be two unblocked reactions. The coupling relationships $\overset{=0}{\to}, \overset{=0}{\leftrightarrow}, \backsim^\lambda$ are defined in the following way:*

- $i \overset{=0}{\to} j$ *if for all $v \in C$, $v_i = 0$ implies $v_j = 0$.*
- $i \overset{=0}{\leftrightarrow} j$ *if for all $v \in C$, $v_i = 0$ is equivalent to $v_j = 0$.*
- $i \backsim^\lambda j$ *if there exists $\lambda \in \mathbb{R}$ such that for all $v \in C, v_j = \lambda v_i$.*

Obviously, $i \backsim^\lambda j$ implies $i \overset{=0}{\leftrightarrow} j$. Moreover, $i \overset{=0}{\leftrightarrow} j$ is equivalent to ($i \overset{=0}{\to} j$ and $j \overset{=0}{\to} i$). The next results shows that the relations $i \overset{=0}{\to} j$, $i \overset{=0}{\leftrightarrow} j$, $i \backsim^\lambda j$ cannot hold for arbitrary pairs of reactions.

Theorem 2. *Let i, j be two unblocked reactions such that at least one of the relations $i \overset{=0}{\to} j$, $i \overset{=0}{\leftrightarrow} j$ or $i \backsim^\lambda j$ is satisfied. Then either (a) or (b) holds:*

(a) *i and j are both (pseudo-)irreversible: $i, j \in Irev \cup Prev$.*
(b) *i and j are both fully reversible: $i, j \in Frev$.*

In the following, we study the coupling relationships for the different types of reactions. We first consider the case $i \in Prev$.

Proposition 2. *Suppose i, j are unblocked, $i \in Prev$ and $j \in Irev \cup Prev$. Then the following are equivalent:*

1. $i \overset{=0}{\to} j$
2. $i \overset{=0}{\leftrightarrow} j$
3. $i \backsim^\lambda j$
4. $g^k_j = \lambda g^k_i$, *for all $k = 1, \ldots, s$.*

In each of these cases, $j \in Prev$.

Next, we characterize the case $i \in Frev$.

Proposition 3. *Suppose i, j are unblocked and $i \in Frev$ is fully reversible. Then the following are equivalent:*

1. $i \overset{=0}{\to} j$
2. $i \overset{=0}{\leftrightarrow} j$
3. $i \backsim^\lambda j$
4. $g^k_j = \lambda g^k_i$, *for all $k = 1, \ldots, s$, and $b^l_j = \lambda b^l_i$, for all $l = 1, \ldots, t$.*

In each of these cases, $j \in Frev$.

Finally, we have to consider $i \in Irev$.

Proposition 4. *Suppose i, j are unblocked, $i \in Irev$ and $j \in Irev \cup Prev$. Then the following are equivalent:*

1. *$i \stackrel{=0}{\rightarrow} j$ holds in the flux cone C.*
2. *$i \stackrel{=0}{\rightarrow} j$ holds in all minimal proper faces $G^k, k = 1, \ldots, s$.*
3. *$g_i^k = 0$ implies $g_j^k = 0$, for all $k = 1, \ldots, s$.*

If also $j \stackrel{=0}{\leftrightarrow} i$ or $i \backsim^{\lambda} j$, then $j \in Irev$.

Corollary 1. *Suppose i, j are unblocked and $i, j \in Irev$. Then we have:*

a) *$i \stackrel{=0}{\leftrightarrow} j$ iff $g_i^k = 0$ is equivalent to $g_j^k = 0$, for all $k = 1, \ldots, s$.*
b) *$i \backsim^{\lambda} j$ iff $g_j^k = \lambda g_i^k$, for all $k = 1, \ldots, s$.*

Table 1 summarizes the different possible coupling relationships. Note that $i \stackrel{=0}{\rightarrow} j$, $i \stackrel{=0}{\leftrightarrow} j$ and $i \backsim^{\lambda} j$ are equivalent for $i, j \in Prev$ or $i, j \in Frev$.

	Irev			*Prev*			*Frev*		
i/j	$\stackrel{=0}{\rightarrow}$	$\stackrel{=0}{\leftrightarrow}$	$\backsim^{\lambda}$	$\stackrel{=0}{\rightarrow}$	$\stackrel{=0}{\leftrightarrow}$	$\backsim^{\lambda}$	$\stackrel{=0}{\rightarrow}$	$\stackrel{=0}{\leftrightarrow}$	$\backsim^{\lambda}$
Irev	Prop.4	Cor.1	Cor.1	Prop.4					
Prev				Prop.2	Prop.2	Prop.2			
Frev							Prop.3	Prop.3	Prop.3

Table 1. Reaction coupling cases

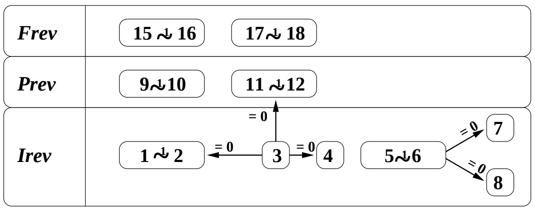


Fig. 3. Coupled reactions in ILLUSNET

Example 3. Fig. 3 shows all coupled reactions in the network from Fig. 2. We see that many reactions depend on reaction 3. A zero flux for this reaction implies a zero flux for the reactions $1, 2, 4, 11$ and 12. Thus, reaction 3 plays a crucial role in the network. Reaction 19 is blocked, because it is involved neither in the definition of the minimal proper faces nor in the definition of the lineality space.

4 Identifying blocked and coupled reactions

4.1 Improving the FCF prodecure

It follows from Sect. 3 that coupling relationships depend on the reversibility type of the reactions. Irreversible and pseudo-irreversible reactions cannot be coupled with fully reversible reactions. According to Table 1, to detect coupling relationships, we do not have to explore exhaustively all possible reaction pairs. We can improve the FCF procedure significantly by applying linear-fractional programming only in those cases where coupling relationships can occur. All the possible cases are given in Table 1. An empty entry indicates that the corresponding coupling relationship is not possible. Note that the reversibility type of a reaction can be identified without computing a set of generating vectors of the flux cone. However, this improved version of FCF still requires a network reconfiguration, which leads to a large number of linear optimization problems that have to be solved.

4.2 A new algorithm

The results in Sect. 3 also suggest a new algorithm to identify blocked and coupled reactions. This method does not require any reconfiguration of the metabolic network. It is only based on the reversibility type of the reactions and a minimum set of generators of the flux cone. The basic steps of this new algorithm are as follows. First, we compute a set of generators of the flux cone C using existing software for polyhedral computations. Second, we classify the reactions according to their reversibility type. This classification allows us to determine whether a coupling between two reactions is possible. Finally, we apply the results from Sect. 3 to identify blocked and coupled reactions. For a more detailed description, see Algorithm 1.

4.3 Computational results

Both our new algorithm and the FCF algorithm have been implemented in the Java language. The FCF procedure was realized using CPLEX 9.0 (a state-of-the-art solver for linear and integer programming problems) accessed via Java. To compute a set of generating vectors of the steady state flux cone, our algorithm uses the software *cdd* [2], which is a C++ implementation of the Double Description method of Motzkin et al. for general convex polyhedra in $\mathbb{R}^n$.

To compare the two approaches, we computed blocked and coupled reactions for some genome-scale networks. The computations were performed on a Linux server with a AMD Athlon Processor 1.6 GHz and 2 GB RAM. We present computation times for models of the human red blood cell [13], the human cardiac mitochondria [12], the central carbon metabolism of *E. coli* [3, 10], the *E. coli K-12* (iJR904 GSM/GPR) [7], and the *H. pylori* (iIT341 GSM/GPR) [11]. We refer to [1] for a discussion of the biological aspects of flux coupling analysis.

Input : Sets $Irev, Prev, Frev \subseteq \{1, \ldots, n\}$;
for each minimal proper face $G^k, k = 1, \ldots, s$, a generating vector $g^k \in G^k \setminus \text{lin.space}(C)$;
a vector basis $b^1, \ldots, b^t$ of lin.space(C).
Output: Blocked reactions: $\Phi = \{i \mid i \text{ is blocked}\}$;
Coupled reactions: $A = \{(i,j) \mid i \backsim^{\lambda} j, 1 \leq i < j \leq n\}$,
$B = \{(i,j) \mid i \overset{=0}{\leftrightarrow} j, 1 \leq i < j \leq n, (i,j) \notin A\}$,
$C = \{(i,j) \mid i \overset{=0}{\rightarrow} j, (i,j) \notin A \cup B, (j,i) \notin A \cup B\}$.

Initialization: $\Phi := \emptyset$, $A := \emptyset$, $B := \emptyset$, $C := \emptyset$.

```
/* Blocked reactions */
```

foreach $i \in \{1, \ldots, n\}$ **do** `/* Proposition 1 */`
  if $(b_i^l = 0, \forall l = 1, \ldots, t)$ *and* $(g_i^k = 0, \forall k = 1, \ldots, s)$ **then**
    add(i, Φ);
  end
end

```
/* Coupled reactions */
```

$Irev := Irev \setminus \Phi$, $Prev := Prev \setminus \Phi$;

foreach $i, j \in Prev$ *with* $i < j$ **do** `/* Proposition 2 */`
  if $\exists \lambda \in \mathbb{R}$ *such that* $g_j^k = \lambda g_i^k, \forall k = 1, \ldots, s$ **then** add$((i,j), A)$;
end
foreach $i, j \in Frev$ *with* $i < j$ **do** `/* Proposition 3 */`
  if $\exists \lambda \in \mathbb{R}$ *such that* $b_j^l = \lambda b_i^l, \forall l = 1, \ldots, t,$ *and* $g_j^k = \lambda g_i^k, \forall k = 1, \ldots, s$
  then add$((i,j), A)$;
end
foreach $i \in Irev, j \in Irev \cup Prev$ **do** `/* Proposition 4 */`
  if $g_i^k \neq 0$ *or* $g_j^k = 0, \forall k = 1, \ldots, s$ **then** add$((i,j), C)$;
end
foreach $(i,j) \in C$ *with* $i, j \in Irev$ *and* $i < j$ **do** `/* Corollary 1 */`
  if $(j,i) \in C$ **then**
    remove$((i,j), C)$, remove$((j,i), C)$;
    if $\exists \lambda \in \mathbb{R}$ *such that* $g_j^k = \lambda g_i^k, \forall k = 1, \ldots, s$ **then** add$((i,j), A)$;
    else add$((i,j), B)$;
  end
end

Algorithm 1: Blocked and coupled reactions finder

Tab. 2 summarizes our computational results. It shows that flux coupling analysis can be done extremely fast if a set of generators of the flux cone is available. Computing such a set is the most time-consuming part in our algorithm. However, it should be noted that this step has an interest in its own. We obtain similar information as by computing the elementary flux modes or extreme pathways of the network, see [4] for more information. The overall running time of the new algorithm is still significantly faster than the original FCF method.

Table 2. Metabolic systems, with the number of blocked reactions (*Blk*), the size of the sets *Irev*, *Prev*, *Frev*, the running time (in seconds) of computing a set of generators (*MMB*), reaction coupling using this set (*FCMMB*), and reaction coupling using the FCF procedure (*FCF*).

Metabolic network	Blk	Irev	Prev	Frev	MMB	FCMMB	FCF
Red Blood Cell	0	31	14	6	2.32	0.26	110.65
Central metabolism of *E. coli*	0	92	18	0	214.49	2.55	477.14
Human cardiac mitochondria	121	83	3	9	1262.65	0.34	13426.91
Helicobacter pylori	346	128	15	39	13551.44	0.43	318374.15
E. coli K-12	435	480	49	110	261306.15	5.32	$\geq$ 1 week

5 Conclusion

In this paper, we have introduced a new method for flux coupling analysis based on a refined analysis of the steady state flux cone. By distinguishing three types of reactions (irreversible, pseudo-irreversible, fully reversible), we study mathematical dependencies between coupling relationships and the reversibility type of the reactions. The results that have been obtained allow improving the flux coupling finder (FCF) and lead to a new algorithm for identifying blocked and coupled reactions in a metabolic network.

References

1. A.P. Burgard, E.V. Nickolaev, C.H. Schilling, and C.D. Maranas. Flux coupling analysis of genome-scale metabolic network reconstructions. *Genome Res.*, 14:301–312, 2004.
2. K. Fukuda and A. Prodon. Double description method revisited. In *Combinatorics and Computer Science*, pages 91–111. Springer, LNCS 1120, 1995. Software available: `http://www.ifor.math.ethz.ch/~fukuda/cdd_home/`.
3. S. Klamt and J. Stelling. Combinatorial complexity of pathway analysis in metabolic networks. *Mol. Bio. Rep.*, 29:233–236, 2002.
4. A. Larhlimi and A. Bockmayr. Minimal metabolic behaviors and the reversible metabolic space. Preprint 299, DFG Research Center Matheon, December 2005. `http://page.mi.fu-berlin.de/~bockmayr/MMB.pdf`.

5. J.A. Papin, J. Stelling, N. D. Price, S. Klamt, S. Schuster, and B. O. Palsson. Comparison of network-based pathway analysis methods. *Trends Biotechnol.*, 22(8):400–405, 2004.
6. N. D. Price, J. L. Reed, and B. O. Palsson. Genome-scale models of microbial cells: evaluating the consequences of constraints. *Nat Rev Microbiol.*, 2(11):886–97, 2004.
7. J.L. Reed, T.D. Vo, C.H. Schilling, and B.Ø. Palsson. An expanded genome-scale model of Escherichia coli K-12 (iJR904 GSM/GPR). *Genome Biol.*, 4(9):R54.1–R54.12, 2003.
8. A. Schrijver. *Theory of Linear and Integer Programming.* Wiley, 1986.
9. S. Schuster and R. Schuster. Detecting strictly detailed balanced sub-networks in open chemical reaction networks. *J. Math. Chem.*, 6:17–40, 1991.
10. J. Stelling, S. Klamt, K. Bettenbrock, S. Schuster, and E.D. Gilles. Metabolic network structure determines key aspects of functionality and regulation. *Nature*, 420:190–193, 2002.
11. I. Thiele, T.D Vo, N.D. Price, and B.Ø. Palsson. An expanded metabolic reconstruction of Helicobacter pylori (iIT341 GSM/GPR). *J. Bacteriol.*, 187(16):5818–5830, 2005.
12. T.D Vo, H.J. Greenberg, and B.Ø. Palsson. Reconstruction and functional characterization of the human mitrochondrial metabolic network based on proteomic and biochemical data. *J. Biol. Chem.*, 279(38):39532–39540, 2004.
13. S.J. Wiback and B.O. Palsson. Extreme pathway analysis of human red blood cell metabolism. *Biophys. J.*, 83:808–818, 2002.

Appendix A

Proof of Theorem 2: First suppose $i \in \mathit{Irev} \cup \mathit{Prev}$ and $j \in \mathit{Frev}$. Since $j \in \mathit{Frev}$, there exists $v \in \text{lin.space}(C)$ such that $v_j \neq 0$. Since $i \in \mathit{Irev} \cup \mathit{Prev}$, we have $v_i = 0$, so $i \overset{=0}{\not\to} j$.

Now suppose that $i \in \mathit{Frev}$ and $j \in \mathit{Irev} \cup \mathit{Prev}$. Since j is unblocked, there exists $w \in C$ such that $w_j \neq 0$. Since i is fully reversible, there exists $b \in \text{lin.space}(C)$ such that $b_i \neq 0$. Define $v = w - (w_i/b_i) \cdot b$. It follows $v \in C$, $v_i = 0$ and $v_j = w_j \neq 0$, which implies $i \overset{=0}{\not\to} j$. □

Proof of Proposition 2: (3) ⇒ (2) ⇒ (1) is immediate.

(1) ⇒ (4): Let $K^+ = \{k \mid g_i^k > 0\}$ and $K^- = \{k \mid g_i^k < 0\}$. Since $i \in \mathit{Prev}$, there exist $v^+, v^- \in C$ with $v_i^+ > 0$ and $v_i^- < 0$. If $K^+ = \emptyset$ (resp. $K^- = \emptyset$), we would have $v_i \leq 0$ (resp. $v_i \geq 0$), for all $v \in C$, which is a contradiction. So both K^+ and K^- must be non-empty. Let $p \in K^+$ and $q \in K^-$. Define $w = g_i^p \cdot g^q - g_i^q \cdot g^p$. Then $w \in C$ and $w_i = 0$. Since $i \overset{=0}{\to} j$, we get $w_j = g_i^p g_j^q - g_i^q g_j^p = 0$, or $g_j^p / g_i^p = g_j^q / g_i^q \overset{\text{def}}{=} \lambda$, independently of the choice of p and q. We conclude $g_j^k = \lambda g_i^k$, for all $k \in K^+ \cup K^-$. Since $i \overset{=0}{\to} j$, this holds for all $k = 1, \ldots, s$.

(4) ⇒ (3): For all $v \in C$, there exists $b \in \text{lin.space}(C)$ and $\alpha_k \geq 0$ such that $v = \sum_{k=1}^{s} \alpha_k g^k + b$. Since $i \in \mathit{Prev}$ and $j \in \mathit{Irev} \cup \mathit{Prev}$, we have $b_i = b_j = 0$. It follows that $v_j = \sum_{k=1}^{s} \alpha_k g_j^k = \sum_{k=1}^{s} \alpha_k \lambda g_i^k = \lambda v_i$. □

Proof of Proposition 3: (3) $\Rightarrow$ (2) $\Rightarrow$ (1) and (3) $\Leftrightarrow$ (4) are immediate.

To prove (1) $\Rightarrow$ (3), we suppose $i \overset{=0}{\to} j$. Since i is fully reversible, there exists $b \in$ lin.space(C), with $b_i \neq 0$. Given $v \in C$, define $w = v - (v_i/b_i) \cdot b$. Then $w \in C$ and $w_i = 0$. Since $i \overset{=0}{\to} j$, we get $w_j = v_j - (v_i/b_i)b_j = v_j - (b_j/b_i)v_i = 0$. Defining $\lambda = b_j/b_i$, this shows $v_j = \lambda v_i$, for all $v \in C$. $\square$

Proof of Proposition 4: (1) $\Rightarrow$ (2) $\Rightarrow$ (3) is obvious, so we have to prove only (3) $\Rightarrow$ (1). For all $v \in C$, there exist $b \in$ lin.space(C) and $\alpha_k \geq 0$ such that $v = \sum_{k=1}^{s} \alpha_k g^k + b$. Since $i \in$ *Irev* and $j \in$ *Irev* $\cup$ *Prev*, we get $b_i = b_j = 0$. By the definition of *Irev*, either $g_i^k \geq 0$, for all $k = 1, \dots, s$, or $g_i^k \leq 0$, for all $k = 1, \dots, s$. Suppose $v_i = \sum_{k=1}^{s} \alpha_k g_i^k = 0$. It follows $g_i^k = 0$, for $k = 1, \dots, s$. Using (3), we obtain $g_j^k = 0$ for $k = 1, \dots, s$, and so $v_j = \sum_{k=1}^{s} \alpha_k g_j^k = 0$.

Under the hypotheses of Prop. 4, suppose $i \overset{=0}{\leftrightarrow} j$. Clearly, $j \overset{=0}{\to} i$. If $j \in$ *Prev*, then by Prop. 2, $i \in$ *Prev*, which is a contradiction. So $j \in$ *Irev*. Similarly, if $i \backsim^{\lambda} j$, then $i \overset{=0}{\leftrightarrow} j$, and again $j \in$ *Irev*. $\square$

Proof of Corollary 1: a) Suppose $g_j^k = 0$ is equivalent to $g_i^k = 0$, for all $k = 1, \dots, s$. Then $g_i^k = 0$ implies $g_j^k = 0$, and vice versa, for all $k = 1, \dots, s$. Since $i, j \in$ *Irev*, we may apply Prop. 4 and get $i \overset{=0}{\to} j$ and $j \overset{=0}{\to} i$. So $i \overset{=0}{\leftrightarrow} j$.
b) Suppose $g_j^k = \lambda g_i^k$, for all $k = 1, \dots, s$. For all $v \in C$, there exist $b \in$ lin.space(C) and $\alpha_k \geq 0$ such that $v = \sum_{k=1}^{s} \alpha_k g^k + b$. Since $i, j \in$ *Irev*, we have $b_i = b_j = 0$. It follows that $v_j = \sum_{k=1}^{s} \alpha_k g_j^k = \sum_{k=1}^{s} \alpha_k \lambda g_i^k = \lambda v_i$. $\square$