Novel Methods for Analysis of Heterogeneous Protein-Cell Interactions

Resolving How the Epidermal Growth Factor Binds to Its Receptor

HANNA BJÖRKLUND
Abstract


Cells are complex biological units with advanced signalling systems, a dynamic capacity to adapt to its environment, and the ability to divide and grow. In fact, they are of such high level of complexity that it has deemed extremely difficult or even impossible to completely understand cells as complete units. The search for comprehending the cell has instead been divided into small, relatively isolated research fields, in which simplified models are used to explain cell biology. The result produced through these reductionistic investigations is integral for our current description of biology. However, there comes a time when it is possible to go beyond such simplifications and investigate cell biology at a higher level of complexity. That time is now.

This thesis describes the development of mathematical tools to investigate intricate biological systems, with focus on heterogeneous protein interactions. By the use of simulations, real-time measurements and kinetic fits, standard assays for specificity measurements and receptor quantification were scrutinized in order to find optimal experimental settings and reduce labour time as well as reagent cost. A novel analysis platform, called Interaction Map, was characterized and applied on several types of interactions. Interaction Map decomposes a time-resolved binding curve and presents information on the kinetics and magnitude of each interaction that contributed to the curve. This provides a greater understanding of parallel interactions involved in the same biological system, such as a cell. The heterogeneity of the epidermal growth factor receptor (EGFR) system was investigated with Interaction Map applied on data from the instrument LigandTracer, together with complementing manual assays. By further introducing disturbances to the system, such as tyrosine kinase inhibitors and variation in temperature, information was obtained about dimerization, internalization and degradation rates.

In the long term, analysis of binding kinetics and combinations of parallel interactions can improve the understanding of complex biomolecular mechanisms in cells and may explain some of the differences observed between cell lines, medical treatments and groups of patients.

Keywords: Heterogeneity, Kinetics, EGFR, HER2, LigandTracer, Interaction Map, Internalization, Specificity

Hanna Björkelund, Uppsala University, Department of Radiology, Oncology and Radiation Science, Biomedical Radiation Sciences, Akademiska sjukhuset, SE-751 85 Uppsala, Sweden.

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Till er som brinner
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Related Work Not Included in This Thesis


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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1:1 model</td>
<td>One-to-one interaction model, describing one monovalent Ligand binding to one Target</td>
</tr>
<tr>
<td>$^{125}\text{I}$</td>
<td>Iodine-125, a radioisotope used for labeling</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ErbB1</td>
<td>Epidermal growth factor receptor type 1, EGFR</td>
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<tr>
<td>HER1</td>
<td>Epidermal growth factor receptor type 1, EGFR</td>
</tr>
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<td>Human epidermal growth factor receptor type 3</td>
</tr>
<tr>
<td>HER4</td>
<td>Human epidermal growth factor receptor type 4</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IM</td>
<td>Interaction Map</td>
</tr>
<tr>
<td>$k_a$</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>$k_d$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>L</td>
<td>Ligand</td>
</tr>
<tr>
<td>LT</td>
<td>Ligand-Target complex</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
</tr>
<tr>
<td>$S_{\text{max}}$</td>
<td>Maximum binding signal</td>
</tr>
<tr>
<td>T</td>
<td>Target</td>
</tr>
<tr>
<td>$T_{\text{tot}}$</td>
<td>Total number of Targets</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
</tbody>
</table>
Introduction

About this thesis
Proteins are biological macromolecules that are essential for life as we know it. They serve as structural components in cells and are involved in almost all biological processes. No matter if their function is catalytical (enzymes), DNA triggering (transcription factors) or involved in the immune response (antibodies), proteins typically interact with other molecules in order to perform their tasks [1]. The characterization of protein interactions is therefore an important part of cell-biology research.

This thesis describes the use of real-time measurements to analyze protein interactions in detail. A key factor has been the access of the instrument LigandTracer®, which can continuously detect the binding of molecules to cells. Data from such real-time measurements provide information about association and dissociation rates, which are highly relevant when translating the results from interaction measurements into hypothesis of the biological effect in an animal or human [2]. New tools were created that decipher time resolved data into information about interaction heterogeneity.

Apart from the development of analysis tools, the thesis further describes how the tools were implemented in the investigation of the interaction between the epidermal growth factor (EGF) and its receptor (EGFR). Overexpression of EGFR has been linked to a number of cancers, which has made it important to learn more about the interaction, how it varies between patients and cell types and how it responds to drugs.

The combination of instrument technology, mathematics and cell biology is relatively unusual. The purpose of this Introduction section is to provide you, the reader, with guidance for comprehending the findings of this PhD project. It starts with a short mathematical description and continues with some background information about the biology.

Interaction models in biology
The simplest model of a biomolecular interaction is the 1:1 (“one-to-one”) model, where one Ligand (L) binds to one Target (T), forming a complex. The interaction is typically reversible and is maintained by weak forces such as ionic bonds, hydrogen bonds and van der Waals forces [1]. The word
Ligand is often used for a small molecule or peptide that triggers a biological process by binding to a specific site on the larger Target protein. In this text, the term Ligand is used in a wider sense and may be anything from an antibody (binding to its target antigen) to a small synthetic molecule (binding to e.g. a cell-surface receptor). In interaction measurements in this thesis, the Ligand is the free molecule in solution, while the Target is a biomolecule immobilized on a surface or integrated with a cell membrane (Fig. 1).

![Diagram of Ligand (L) binding to Target (T), forming the complex LT. In this thesis, T is immobilized on a surface or anchored to a cell membrane.]

**Equilibrium versus dynamics**

Ligands and Targets can interact once they are close enough. As a result of the reversible nature of the interaction, complexes will simultaneously fall apart. The rate of formation and separation, or association and dissociation, is related to the concentrations of complexes and free Ligand and Target. At the beginning, when there is nothing but free Ligand and Target, the rate of complex formation is at its maximum. Over time, the concentration of free Ligand and Target is reduced as complexes are formed. This causes a decrease in association rate. Simultaneously, the larger number of existing complexes results in more complexes falling apart each time unit. Consequently, the amount of complexes will eventually reach a level where the association and dissociation rates are equal. At this state of equilibrium, the amount of complexes will remain constant as long as the concentrations of Ligand and Target do not change. This is often the case for *in vitro* experiments, but concentrations can fluctuate greatly in living organisms (Fig. 2) [3].
Figure 2. The different stages of an interaction. A) Before Ligand has been added to the Target, no complexes exist. B) At the beginning of the interaction, the association is dominant as dissociation requires existing complexes. C) After some time, the number of complexes forming is equal to the number of complexes falling apart each time unit, i.e. the system is in equilibrium. D) A decrease in free Ligand will make the dissociation rate dominant, resulting in fewer complexes.

Historically, much emphasis has been put on discussing interaction properties at equilibrium, where the equilibrium dissociation constant $K_D$ is used for describing the strength, the affinity, of an interaction. In a biological context however, the effect of an interaction may occur long before equilibrium has been reached. Furthermore, the time for a biological effect to remain is generally associated with for how long the Ligand stays bound, described by the dissociation rate [4]. Therefore, not only the affinity, but also the rate of Ligand association and dissociation, seem to be important. This has been presented in several studies the last decade:

- Markgren et. al. stated the necessity of studying kinetic properties in drug design, rather than affinity alone. The data showed differences in association and dissociation rates of several orders of magnitude between drug leads with the same affinity. [5]
- The estimation of unwanted immunogenicity is a necessary part of the safety evaluation of therapeutic biomolecules. However, studies have shown that low affinity antibodies can trigger the immune response without being detectable in ELISA studies due to their rapid dissociation rates [6, 7].
- The equilibrium dissociation constant is the ratio of the dissociation and association rate. In a QSAR study, Andersson et. al. pin-pointed specific amino acids affecting association and dissociation rates and showed that these were not the same, indicating that association and dissociation are regulated by different parts of the protein structure. Thus, the use of affinity as a parameter to describe an interaction should be done with care as it is a simplification that may be misleading [8].
- The use of incubation times that are too short for reaching equilibrium can cause underestimations of the affinity of several orders of magnitude
[9]. Hulme et. al. showed that an incubation time of at least five times the half time of the equilibration reaction is necessary [10], which corresponds to 10-100 h for a number of common therapeutic antibodies.

These are merely some of the findings that indicate how kinetic information can improve the biological understanding and minimize the risk of false negative results. The focus is slowly shifting from classical equilibrium based measurements towards a more dynamic approach. This is observed not only in published data, but also in a reported growing need of accurate methods for detecting protein interactions [11].

The mathematics behind the kinetics

The reversible 1:1 interaction model can be written as

$$L + T \leftrightarrow LT$$

(1)

where free Ligand L binds to Target T to form the complex LT. The formation over time can be described by the differential equation

$$\frac{d[L,T]}{dt} = k_a \times [L] \times [T] - k_d \times [LT]$$

(2)

where $k_a$ ($M^{-1}s^{-1}$) is the association rate constant and $k_d$ ($s^{-1}$) is the dissociation rate constant describing the formation and separation of the complex [12]. Put simple, the concentrations of free L and T will determine the chances of L and T coming near enough to interact. Once close to each other, the likelihood of L and T forming a complex is determined by $k_a$. The constant $k_d$ describes the stability of the interaction. Some complexes form easily, but may not be stable and will thus fall apart quickly. These kind of interactions are often referred to as fast on – fast off interactions and have large $k_a$ and $k_d$ values. Other complexes may form slowly, but are stable once formed. These are slow on – slow off interactions, with low $k_a$ and $k_d$ values. In the cell, interactions with a wide range of $k_a$ and $k_d$ combinations are possible.

The affinity, or how tightly the two molecules bind, can be described by the equilibrium dissociation constant, $K_D$ (M), where

$$K_D = \frac{[L] \times [T]}{[LT]} = \frac{k_d}{k_a}$$

(3)

$K_D$ corresponds to the concentration of Ligand at which half of the Targets have a Ligand bound to them when the system is in equilibrium, i.e. when the
rate at which complexes are formed is equal to the rate at which they fall apart. Under the assumption that $T_{tot}$, the total amount of Targets (the sum of bound and unbound), is constant over time and that the Ligand is available in large excess to avoid depletion, the amount of formed complexes at a specific Ligand concentration can be calculated from equation 2. This results in

$$[LT] = T_{tot} \times \frac{[L]}{[L]+K_D} \times f(t)$$

(4)

where $f(t) = 1$ at steady-state. This means that if using a Ligand concentration of $0.1 \times K_D$, $1 \times K_D$ or $10 \times K_D$, the amount of bound Targets at equilibrium will be 10 %, 50 % or 91 % respectively.

Before equilibrium is reached the function $f(t)$ describes how the 1:1 interaction changes over time:

$$f(t)_{association} = \left(1 - e^{-k_a \times [L] + k_d \times t}\right)$$

(5)

$$f(t)_{dissociation} = e^{-k_d \times t}$$

(6)

**On-off plots**

Interactions can be summarized and compared graphically using on-off plots, where each interaction is represented by a dot. The kinetic parameters $k_a$ and $k_d$ will define the position of the dot, using logarithmic scales on the axes. As the dissociation constant $K_D$ is the ratio of $k_d$ and $k_a$, the affinity is represented in the plot as well (Fig. 3) [5]. On-off plots are convenient when comparing several similar Ligands, such as drug leads [13].

![Figure 3. Example of an on-off plot. The interactions are plotted as dots on positions representing their $k_a$ and $k_d$ values.](image)
LigandTracer

LigandTracer® (Ridgeview Instruments AB, Uppsala, Sweden) is an instrument developed to monitor Ligand-Target interactions in real-time, focusing on Targets associated with cells (e.g. cell surface receptors). The instrument measures continuously, providing the user with information not only on the affinity of the interaction, but also on the kinetic properties.

Measurements in LigandTracer require Ligands labeled with either radioactivity or fluorescence and Targets that can be immobilized to the plastic surface of a petri dish. In most cases, the latter is obtained by the use of adherent cells, but applications have been developed for anchoring cells in suspension as well. The cells are grown on a specific area of the petri dish, which is put on an inclined, rotating support, followed by addition of labeled Ligand (Fig. 4).

![Figure 4. The principle of the LigandTracer technology.](image)

As the dish rotates, a detector mounted above the upper part of the dish monitors the signal from the area passing by. Any binding of Ligand to the Target will be observed as a signal peak when the Target area is detected. The peak is followed over time, creating a real-time binding curve describing the association and the dissociation of the Ligand (Fig. 5). The non-active plastic area of the dish is used as a reference, to correct for any background signal [14].
Heterogeneous protein interactions

The research field of molecular and cellular biology has historically moved forward on the assumption that complicated biological networks of interactions can be explained by simple models. In fact, most biomolecular interactions are implicitly considered to fit a 1:1 binding model describing a monovalent Ligand binding to a single type of Target (Fig. 6A) simply because the term affinity is used [15]. This may be a rather harsh simplification, as cells tend to be dynamic and complex with a broad range of backup mechanisms if any pathway were to be disrupted [16, 17]. It is possible that there are several versions of the Target, such as different conformations (Fig. 6B) [18], molecular variants (members of the same receptor family, differences in post-translational modifications etc., Fig. 6C) [19], or hetero and homo dimers (Fig. 6D) [20]. The Ligand may bind to these alternative Target variants with different strength, resulting in a spectrum of interactions occurring simultaneously, with a wide range of kinetic and affinity characteristics.

Figure 5. Signal peak height followed over time. A) As more Ligand bind, the signal will increase, observed as a higher peak height. B) The peak heights can be plotted over time to form a binding curve.

Figure 6. Examples of hypothetical variants of Target T, which Ligand L may bind to with different interaction properties: A) the “standard” variant of T, B) different conformations of T, C) molecular variants of T and D) T dimerized with other surface proteins (S.P.).
The possible heterogeneity of interactions is seldom discussed, probably because there is a lack of methods to analyze intricate interactions on a level that reflects the complexity. Most biomolecular interaction assays are still based on end-point measurements, which are often insufficient for a full understanding of the dynamics of an interaction (or a sum of interactions, in the case of heterogeneity). There are some alternatives to the end-point measurements, such as the biosensor Biacore™ (GE Healthcare, Uppsala, Sweden) that is widely used. A Biacore instrument provides information on the kinetics, but it is based on a simplified system containing purified proteins (resembling the situation in Fig. 6A) [21], which may be too far from the potential heterogeneous reality of living cells.

Another explanation of the simplification of biological systems into 1:1 interactions may be that the heterogeneity has not yet been proven important enough to be taken into consideration. However, the impact of heterogeneity will be difficult to monitor without proper analysis tools.

Understanding the heterogeneity of an interaction opens up for many new possibilities. First of all, the design and development of new drugs would likely benefit extensively from a more thorough understanding of the systems of interest [22]. If information about e.g. backup pathways is available in an early stage of development, the step from pure protein – protein interaction measurements to in vivo studies would be smaller, and the risk of spending unnecessary months on drug candidates that in a later stage proves ineffective would be reduced [23, 24]. Secondly, the heterogeneity of an interaction may explain the great variety in patient response to certain treatments. If the clinicians could be given access to a more complex but accurate description of the state of the disease of the patient, the choice of treatment might be chosen more wisely. This way of treating individual patients based on their specific disease morphology, so called personalized medicine, may be essential in the future for curing intricate and variable diseases such as e.g. cancer [25, 26].

**Interaction Map**

The mathematical method Interaction Map (IM) may be a step towards more detailed analysis of biomolecular interactions. The main assumption of the method is that the binding of a Ligand to a Target can be expressed as a sum of monovalent interactions [27, 28], each having a unique combination of association rate constant $k_a$ and dissociation rate constant $k_d$:

$$MeasuredCurve = \sum_{i=1}^{n} \sum_{j=1}^{m} [W_{ij} \times CurveComponent(conc, k_{a}, k_{d})]$$
Each curve component is the result of a monovalent interaction and is defined by the kinetic rate constants and the Ligand concentration used. The components will contribute to the measured curve in different manners, as described by the weighting parameter $W$. This makes it possible to decompose a time-resolved binding curve into a two-dimensional distribution of $k_a$ and $k_d$, where each peak in this modified on-off plot corresponds to a contributing component of the measured curve. The weighting factors are represented as colors or darkness of the peak, where large contributions are represented by warm colors (color-IM) or darkness (greyscale-IM) (Fig. 7). In short, a heterogeneous interaction will be deciphered into the underlying components, each represented by a peak in the Interaction Map.

Figure 7. Example of an Interaction Map. The measured curve depicting a stepwise increase in Ligand concentration (Fig. A, grey curve) is the result of three simultaneous interactions, observed as distinct peaks in the map (Fig. B: areas C, D and E). The corresponding simulated binding curves of each peak are presented to the right (Fig. C, D, E). Note that the saturation, or darkness, of the peaks correspond to the degree of contribution. Interaction Maps can also be presented in color, where warm colors represent large contributions. The Ligand in this example was $^{125}$I-labeled epidermal growth factor (EGF).

Cancer

As the life expectancy continue to increase all over the world a new problem has emerged that is strongly associated with high age: cancer. About one third of us will receive a cancer diagnosis during our lifetime [29]. Although progress has been made in the development of new drugs and treatments,
cancer is still a leading cause of death worldwide and continues to increase as a global threat [30, 31].

Unlike infectious diseases, such as flu, malaria and the common cold, the cause of cancer cannot be easily defined as a single external exposure. Instead, cancer is often the result of several factors combined, such as e.g. inherited genetic traits, radiation, chemicals and virus infections [32, 33]. This complexity is further reflected in the nature of the disease. There are approximately 200 different types of cancer, classified by the organ and cell type from which the tumor origins, and each of these can show great diversity between patients [34, 35]. It is therefore difficult to discuss cancer as a single disease in regards to cause, origin or morphology. Cancer is instead defined by its behavior [36, 37]. Common to all cancers are dysregulation in proliferation and apoptosis in endogenous cells, which cause the cells to grow and divide in an uncontrolled manner. For each division the malfunctioning traits are automatically passed on to the new cells, resulting in an exponentially growing tumor and subsequent invasion to other parts of the body through the bloodstream or lymphatic system [36].

One major issue when developing cancer treatments is how to separate the cells with dysregulated growth from the normally functioning cells of the body. In chemotherapy this is accomplished by solely killing dividing cells, with the hope of eradicating the fast-multiplying cancer cells before inflicting too much damage to the healthy tissue. Unfortunately and not too surprisingly, this causes severe side effects and the treatment is therefore restricted by how much the patient can handle [35]. To avoid this, more intricate methods have been developed which target individual molecules typically associated with a certain cancer [38, 39]. However, the inherent heterogeneity of the disease makes the development of such targeted therapy problematic and there may be large differences in how patients with the same cancer type respond to a drug [40-43]. A better understanding of the chosen targets and their variety among patients would thus greatly benefit cancer drug development.

The EGF receptor family

The epidermal growth factor receptor (EGFR) family is a group of receptor tyrosine kinases that are part of an advanced signaling system, where interactions with Ligands trigger cell growth, proliferation and anti-apoptotic activity [44]. The family consists of four members: EGFR (also denoted ErbB1 or HER1), HER2, HER3 and HER4. These receptors and their associated signaling networks are of great importance in e.g. organogenesis, immune responses and embryogenesis and are under normal circumstances strictly regulated. Exceptions to this regulation have been found in several types of cancer, where mutations increasing the expression or activity of
EGFR family members, especially EGFR, are associated with tumor progression [45]. This has made the EGF receptor family an important target for cancer therapy and has in the process become widely studied. Unfortunately, the system is complex and despite extensive investigations many questions remain about its involvement in diseases and how to disrupt their signaling.

Dimerization and activation of EGFR

The members of the EGFR family are all integrated with the cell membrane and consist of an extracellular Ligand binding domain, a transmembrane domain and an intracellular cytoplasmic domain including a tyrosine kinase domain. For HER2, no natural occurring Ligand has been identified and it is considered an orphan receptor. In contrast, HER3 has a normally functional Ligand binding domain but lacks intracellular tyrosine kinase activity [46].

The EGFR family receptors pass signaling from the exterior of the cell to the interior through dimerization. In the absence of signaling Ligands, the receptors exist on the surface in inactive conformations. Upon Ligand binding the receptors undergo a transition to an open, active, conformation where the dimerization arm is exposed. This enables them to interact with other active receptors. Once dimerized, the receptors can cross-phosphorylate tyrosines in the intracellular domain of their dimerization partner, which in turn activates downstream signaling molecules interacting with the phosphorylated residues [46-48].

There are some exceptions to this mechanism. HER2, lacking natural Ligands, have a constitutively exposed dimerization arm [49]. Its active state makes it a suitable dimerization partner for other members of its family, but the receptor can also spontaneously form catalytically active homodimers [50].

Despite the established dimerization and activation model described above, the correlation of Ligand binding to EGFR and further signaling has been discussed for decades and is yet to be fully understood [51]. Data from several recently published studies reveal the presence of Ligand independently formed EGFR dimers (e.g. EGFR – EGFR and EGFR – HER2) on the cell surface [52, 53] (Fig. 8). However, the extent of their existence and effect on downstream signaling has not been completely established.
Figure 8. Conformation and dimer variants of EGFR. A) When no Ligand is bound EGFR exists in an inactive conformation. B) Ligand binding causes a conformation change into an active state where the dimerization arm is exposed, making it possible to form dimers with another active EGFR (homodimer), which results in cross-activation through phosphorylation. C) Active EGFR can dimerize with another member of the EGFR family, here represented by HER2 which exists in a Ligand independent active form. D-E) There are indications of Ligand independent pre-formed EGFR dimers, although the mechanism behind these is not fully established.

Internalization of EGFR

The interaction with EGF and subsequent dimerization causes the active receptors to internalize rapidly through endocytosis. The complexes are separated in an early stage, which inactivates EGFR. The destiny of the receptor is determined in sorting endosomes, where they are either transferred to lysosomes for degradation or returned back to the surface as a recycling procedure [54-56].

Upon dimerization with HER2, the internalization of EGFR can be slow or completely disrupted according to some studies [57-59]. Others report that HER2 heterodimerization have no great impact on early internalization processes, but rather affects the following internal degradation events. These studies show that EGFR dimerized with HER2 are more likely to dissociate in early endosomes and return back to the cell surface as recycled receptors [56, 59].

Tyrosine kinase inhibitors

The strong association with malignant tumors has made EGFR an interesting target for cancer therapy. The strategies used are everything from EGFR-binding antibodies with radioactive nuclides, to inhibitors for the tyrosine kinase domain. The drugs gefitinib (also denoted Iressa™ or ZD1839), lapatinib (Tykerb™), AG1478 and erlotinib (Tarceva™) are examples of EGFR tyrosine kinase inhibitors (TKIs) designed for blocking further downstream signaling [60-64]. The result is typically inhibited growth, but TKI sensitivi-
ty varies between cell lines and patients [61, 65]. Some mutations have been identified as predictive markers for sensitivity or resistance [66, 67], but questions remain about the detailed mechanisms of TKIs and their ability to reduce tumor growth [68, 69].

In addition to reduced growth rate, several other effects of TKIs have been observed. Gefitinib and AG1478 can affect the extracellular interaction with EGF, detected as an increased affinity [70, 71], even though they bind to the intracellular part of EGFR. This may or may not be associated with the ability to form non-active EGFR dimers, which has been observed for gefitinib, AG1478 and erlotinib, but not for lapatinib [70, 72-75]. Furthermore, gefitinib has been reported to reduce internalization rate and slow down the following degradation [69].
Scientific objectives

The aim of this PhD project was to develop and apply methods for the analysis of complex, heterogeneous protein interactions, using the EGF – EGFR interaction as a model for the general case.

This was divided into following sub-goals:

- Utilize kinetics for a theoretical understanding of protein interactions and adapt this knowledge to investigate strengths and weaknesses of common biological measurements.
- Evaluate Interaction Map as an analytical tool to describe interaction heterogeneity.
- Study the heterogeneity of the EGF – EGFR interaction in tumor cells.
- Investigate how EGFR dimerization partners and the presence of tyrosine kinase inhibitors affect binding of EGF.
- Develop a strategy to understand protein interactions in cells at physiological temperatures.

In short, this was not strictly a biological project. Nor was it solely a development of methods. This was an effort to combine biology, mathematics, technology and programming to not only create a tool kit, but also understand the protein interactions to which it was applied.
The present study

Cell lines

Four tumor cell lines were used in this study: The human squamous carcinoma cell line A431, the human glioma cell line U343MGaCl2:6 (a subclone of U343MG [76], denoted U343), the human ovarian carcinoma cell line SKOV3 and the human breast cancer cell line SKBR3.

The cell lines were chosen to cover a wide span of EGFR and HER2 expressions. The numbers of receptors were determined manually or in LigandTracer using the kinetic extrapolation method, as described in Paper II and V (Table 1).

Table 1. EGFR and HER2 expression in A431, U343, SKOV3 and SKBR3 cells cultivated in complete medium. The data, which is presented in Paper V, was generated using the KEX method described in Paper II.

<table>
<thead>
<tr>
<th></th>
<th>A431</th>
<th>U343</th>
<th>SKOV3</th>
<th>SKBR3</th>
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<tbody>
<tr>
<td>EGFR</td>
<td>2.1±0.4E6</td>
<td>6.4±0.5E5</td>
<td>3.4±0.6E5</td>
<td>4.1±0.3E5</td>
</tr>
<tr>
<td>HER2</td>
<td>1.5±0.1E5</td>
<td>3.1±0.6E4</td>
<td>2.0±0.3E7</td>
<td>5.8±0.5E6</td>
</tr>
</tbody>
</table>

Paper I

Aim

Competition measurements are common when establishing the specificity of biological interactions. In these experiments the signal of labeled Ligand in the presence of a large excess of unlabeled Ligand is measured. The amount of unlabeled Ligand is assumed large enough to “block” all Targets. If a high signal is detected despite the blocking of Targets, the interaction is considered unspecific.

The aim of this paper was to investigate some of the common assumptions of specificity measurements and establish how the experimental design can be optimized to minimize the risk of false negative results. Parameters such as pre-incubation, incubation time, concentration of unlabeled Ligand and the impact of reversibility were investigated.
Results

Simulations of protein interactions to understand the impact of concentration, kinetic properties and incubation times

A set of interactions, with a number of $k_a$ and $k_d$ combinations, were simulated in the software MATLAB. The concentrations of unlabeled Ligand were varied and three experimental approaches were tested, simulating i) simultaneous incubation of labeled and unlabeled Ligand (Fig. 9), ii) pre-incubation of unlabeled Ligand, or iii) the addition of unlabeled Ligand to disrupt the interaction.

It was found that the concentration of unlabeled Ligand in relation to $K_D$ is more important than the ratio of labeled and unlabeled Ligand. A concentration of $10 \times K_D$ ensures that almost all receptors are in complex with the unlabeled Ligand, enabling accurate specificity estimation. Therefore, the assumption that e.g. “a 100 times excess of unlabeled Ligand (in relation to labeled Ligand) is enough” is incorrect at low concentrations and is a waste of reagents at high concentrations. Furthermore, a pre-incubation of unlabeled Ligand is beneficial, especially for slow interactions and for low concentrations. An incubation time long enough to reach equilibrium is recommended as well, to clearly separate between blocked and unblocked receptor signals. Disrupting a binding by adding unlabeled Ligand can give indications on the specificity, but the data are more difficult to interpret. Finally, a good understanding of the reversibility of most biological interactions is essential when designing a suitable assay setup.

Real-time protein measurements to confirm conclusions from the theoretical approach

The conclusions drawn from the simulations were tested experimentally by monitoring the $^{125}$I-EGF – EGFR interaction in the human squamous carcinoma cell line A431 in real-time using LigandTracer. The binding of either 0.3 or 30 nM $^{125}$I-EGF, corresponding to $0.1 \times K_D$ or $10 \times K_D$ [71], was measured in the presence of 10 times higher concentration of unlabeled EGF. Experiments were designed according to the three experimental approaches described above.

Results show that 300 nM unlabeled EGF is enough to bind most EGFR, as observed by the low signal from 30 nM $^{125}$I-EGF (Fig. 10 A and C). In contrast, 3 nM unlabeled EGFR did not affect the interaction of 0.3 nM $^{125}$I-EGF much (Fig. 10 B and D). This illustrates that a ratio of labeled and unlabeled Ligand of 1:10 is enough as long as the concentration of unlabeled Ligand is at least $10 \times K_D$. Pre-incubation is beneficial for slow interactions, as seen for the low set of concentrations (Fig. 10 D).
Figure 9. Simulated binding curves of 1 nM labeled Ligand in the presence of 10 nM (filled circles), 100 nM (filled triangles) or 1000 nM (crosses) of unlabeled ligand, added simultaneously to the target. $S_{\text{max}}$, the maximum signal, was set to 100, and different kinetic properties was chosen: A) $k_a = 10^4$, $k_d = 10^{-3}$ ($K_D = 100$ nM), B) $k_a = 10^4$, $k_d = 10^{-4}$ ($K_D = 10$ nM), C) $k_a = 10^5$, $k_d = 10^{-3}$ ($K_D = 10$ nM), D) $k_a = 10^4$, $k_d = 10^{-5}$ ($K_D = 1$ nM), E) $k_a = 10^5$, $k_d = 10^{-4}$ ($K_D = 1$ nM) and F) $k_a = 10^5$, $k_d = 10^{-5}$ ($K_D = 0.1$ nM).
Figure 10. A and C) The binding of 30 nM $^{125}$I-EGF to cultured A431 cells in the presence (triangles) or absence (circles) of 300 nM unlabeled EGF. B and D) The binding of 0.3 nM $^{125}$I-EGF to A431 in the presence (triangles) or absence (circles) of 3 nM unlabeled EGF. Two of the three tested approaches are presented: A-B) Approach 1, simultaneous addition, C-D) Approach 2, pre-incubation of unlabeled Ligand.

Discussion

Spending extra time and energy on assay design can be a key to success in biological research. The findings in this paper demonstrate this. With a few guidelines and a general understanding of the system to be studied, poor data can be avoided. This paper focused on avoiding false negative results in specificity measurements. These types of measurements are performed as a standard routine, but the experimental settings vary greatly between labs.

Experience from contacts with a number of research groups is that too little time is spent on optimizing established protocols, even though the protocols may have been developed for a completely different interaction. In the best case, this will only result in an unnecessary high reagent cost. In the worst case the binding data will indicate unspecific binding even though it is not, which may stop the development of interesting new binders.

The work presented in this paper is an example of how theoretical knowledge about the basics of kinetics can successfully be applied to biological data and provide valuable information in the development or improvement of assays.
Paper II

Aim

Quantification of the number of receptors per cell is particularly important in the search of suitable tumor drug targets and biomarkers. A common method for receptor quantification is the classical manual saturation technique, where an increasing concentration of radiolabeled receptor binding compound is added until all targets are bound. The number of receptors per cell can then easily be calculated from the cell count and specific activity. The method is straightforward, but may require much labor time and large consumptions of reagents.

The aim of this paper was to test a novel method called kinetic extrapolation (KEX) and compare it with the manual saturation procedure regarding accuracy and costs. By detecting the binding of the radiolabeled compound in real-time and then fitting the data with a kinetic model, the maximum signal can be estimated. This way, the need of actually saturating all receptors is circumvented.

Results

The KEX method reduced reagent consumption and work load while maintaining accuracy

EGFR and/or HER2 levels were quantified in five human cell lines using \(^{125}\)I-labeled cetuximab or EGF for EGFR estimation, and trastuzumab for HER2. For the manual saturation method, the binding of antibody/EGF concentrations ranging from 0.5 to 150 nM was measured in triplicates. Cells were incubated for 4 h on ice before cell count and estimation of radioactivity. For the KEX method, a stepwise increase of antibody/EGF concentration (most often 3, 15 and 30 nM) was monitored in LigandTracer for long enough to approach equilibrium. Cell number and activity was then quantified just like for the manual method. Maximum signal level, \(S_{\text{max}}\), was estimated by fitting the LigandTracer data to a kinetic model, in order to compensate for non-saturation of receptors (Fig. 11).
Figure 11. Example of the kinetic evaluation of the KEX method. The binding curves of the $^{125}$I-cetuximab – EGFR (upper black solid curve) and $^{125}$I-EGF – EGFR (lower grey solid curve) interactions were fitted using kinetic models (dotted upper curve and black lower curve). The $S_{\text{max}}$ value, derived from the kinetic fit, corresponds to full EGFR saturation and was used to correct for non-saturation.

Figure 12. Receptor count estimated by the KEX method (open symbols) and the classical manual saturation technique (filled symbols). Results provided from the two methods are comparable, although the variability is somewhat higher for the KEX method.

The results were comparable regarding receptor number, but the KEX method had a slightly higher variability (Fig. 12). In some cases, the KEX method estimated higher receptor quantities than the classical saturation technique.
Antibody consumption using KEX was merely 26-46 % of the amount used in the classical method. At the same time, the work load was reduced by 60 %.

Discussion
The results in this paper indicate that KEX is a reliable method for cell-surface receptor quantification. It reduces manual workload and reagent costs, which makes it a suitable alternative to the standard saturation technique.

The fact that the KEX method sometimes resulted in a higher receptor count is likely due to too short incubation times for the manual technique. The manual technique is based on end-point measurements, which means that it does not provide information on whether equilibrium has been reached or not. There will be an underestimation of the receptor quantity if the incubation time is too short for equilibrium to be established.

The KEX method has been further validated in a separate paper [77].

Paper III
Aim
Complex heterogeneous biological interactions require advanced analysis platforms to understand them. The investigation of e.g. intricate cellular receptor systems has suffered from the lack of such analysis tools. Interaction Map, which deciphers contributing components of a measured heterogeneous interaction from real-time data, may be a step towards grasping the level of complexity often found in biology.

In Paper III we investigated the potential and accuracy of Interaction Map by applying it to artificially generated heterogeneous data with known interaction components.

Results
Interaction Map applied on an artificially generated heterogeneous SPR system to estimate accuracy
Peptides corresponding to residues 138-149 (denoted P138-149) and 140-151 (P140-151) of the tobacco mosaic virus protein were immobilized to a Biacore™ sensor chip at different ratios (Exp. 1: 100 % P138-149, Exp. 2: 75 % P138-149, 25 % P140-151, Exp. 3: 25 % P138-149, 75 % P140-151, Exp. 4: 100 % P140-151). The interaction between the peptides and Fab57P [78] was monitored in a Biacore 2000 instrument, using a concentration series of Fab57P (Fig. 13). Curve data (black curves, left column) were fitted
(grey curves, left column) using regression analysis, with Langmuir model for Exp. 1 and 4 and Parallel Reactions model for Exp. 2 and 3, or with Interaction Map (right column). The corresponding curves for interaction 1 (black curves, central column) and 2 (grey curves, central column) were calculated from the peak areas in the Interaction Maps.

Figure 13. Evaluation of SPR data by regression analysis and Interaction Map. A two-fold dilution series of Fab57P (highest concentration 28 nM) was applied to surfaces carrying A) 100% P138-149 (Exp. 1) B) 75% P138-149, 25% P140-151 (Exp. 2), C) 25% P138-149, 75% P140-151 (Exp. 3) or D) 100% P140-151 (Exp. 4). Left column depicts the measured data (black curves) and regression analysis fits (grey curves), using either the Langmuir model or the Parallel reactions model. Right column presents the calculated Interaction Maps for each experiment. Central column shows the corresponding curves for interaction 1 (black curves) and 2 (grey curves), calculated from the peak areas in the Interaction Maps.

Results from the Langmuir model showed that the Fab57P – P138-149 interaction is 14 times weaker than the Fab57P – P140-151 interaction. The two
Interactions are observed as separate peaks in the Interaction Maps (Fig. 13: “Int. 1” and “Int. 2, right column). Data from Interaction Map calculations were in agreement with the results from regression analysis and the contribution of interaction 1 to interaction 2 shifted according to how the ratios of peptides were changed. Regression analysis only provided accurate results if the starting guesses of the $k_d$ values were set to $10^{-4}$ or $10^{-3}$ s$^{-1}$ and a model was chosen that corresponded to what was known about the artificial systems (i.e. number of parallel interactions). Interaction Map resolved the two contributing interactions without knowledge about kinetic constants or degree of heterogeneity.

**Interaction Map to decipher interaction components with similar binding properties, as exemplified using LigandTracer**

Interaction Map was further evaluated on LigandTracer data depicting the $^{125}$I-HSA – mAb 18080 and $^{125}$I-trastuzumab – Protein A interactions, either separately (Exp 5: Fig. 14 A and C and Exp 8: Fig 14 E and F) or in combination, where changes in specific activity shifted the contribution of the HSA and trastuzumab interactions. In Experiment 6, the $^{125}$I-HSA – mAb 18080 corresponded to 65 % of the measured signal and in Experiment 7, 47 % (Fig. 14. B and D).

![Figure 14](image)

*Figure 14.* Interaction Maps of the $^{125}$I-HSA – Ab 18080 and $^{125}$I-trastuzumab – Protein A interactions as detected in LigandTracer Grey. By applying different concentrations, as well as shifting the specific activity of trastuzumab, a system of HSA/trastuzumab combinations were created, using A) 100 % HSA, 0 % trastuzumab (Exp. 5), B) 65 % HSA, 35 % trastuzumab (Exp. 6), D) 47 % HSA, 53 % trastuzumab (Exp. 7) and E) 0 % HSA, 100 % trastuzumab (Exp. 8). Corresponding LigandTracer data describing the pure C) HSA (Exp. 5) and F) trastuzumab (Exp. 8) interactions are included.

In both the HSA and the trastuzumab measurements, one major ($i$) and one minor ($ii$) peak were observed. The major peak corresponded to an interac-
tion component with similar dissociation rates in all measurements ($\log(k_d) = -5.48 \pm 0.09$), but different association rates. Going from a pure HSA state to a pure trastuzumab state was observed as a gradual shift of the $\log(k_a)$ value of peak $i$, from 4.12 in Exp. 5, to 3.92, 3.75 and 3.62 in Exp. 6, 7 and 8 respectively. The interaction change was further reflected in degree of heterogeneity, where peak $i$ contributed to 66% of the measured signal in the pure $^{125}$I-HSA – mAb 18080 measurement (Exp. 5), but increased upon reduction of the HSA – mAb 18080 contribution, to 70% (Exp. 6), 80% (Exp. 7) and 88% (Exp. 8).

Discussion
The purpose of this paper was to investigate the capacity of Interaction Map, regarding the ability of distinguishing between separate interactions and the accuracy of the estimations of kinetic properties. By comparing results from regression analysis and Interaction Maps of SPR data, it was clear that Interaction Map has the potential to resolve heterogeneous data in an accurate manner even without any information on interaction kinetics or degree of heterogeneity.

The LigandTracer model system was more complex than the SPR model system, with a relatively small variation between the two interactions. The fact that it still was possible to observe the peaks shift in position and contribution demonstrates that Interaction Map can distinguish between interactions even with similar binding properties.

Paper IV and V
Aim
The aim of the work presented in paper IV and V was to investigate the complexity of the interaction between the epidermal growth factor EGF and its receptor (EGFR) by real-time measurements. This was conducted in a variety of cell lines and culturing environments in order to better grasp how cell context affects interaction kinetics. The real-time binding data, in combination with complementary assays, made it possible to form a hypothesis about the EGF – EGFR interaction and EGFR dimerization patterns. The data was further analyzed with the Interaction Map tool to strengthen the hypothesis.

The EGFR family is a suitable model system for the analysis of heterogeneous protein interactions as the members in the family are known to dimerize, forming a complex signaling system. Thanks to intensive research, many details about the interaction have already been apprehended, facilitating interpretations of the binding data presented in these papers.
Results

Real-time measurements in LigandTracer displayed a heterogeneous and cell context dependent EGF – EGFR interaction

The affinity and kinetics of the $^{125}$I-EGF – EGFR interaction were followed in real-time in the four human tumor cell lines A431, U343, SKOV3 and SKBR3, cultured in either complete or serum free medium, in the presence or absence of 1 µM of the TKI gefitinib, using LigandTracer Grey. Increasing concentrations of EGF were used to gather as much information as possible about the kinetics of the interaction. The EGF concentrations had to be chosen differently due to large differences in affinity, as described below.

Culturing conditions had an impact on the $^{125}$I-EGF – EGFR interaction. Gefitinib increased the affinity of the interaction in A431 and SKOV3 cells, observed as a slower dissociation rate and less increase in signal upon addition of the second EGF concentration. This effect was amplified in A431 cells grown in serum free medium. U343 was sensitive to starvation (i.e. serum free medium), which decreased the affinity of the interaction. SKBR3 seemed to be insensitive to all of the tested treatments (Fig. 15).

Figure 15. The effect of gefitinib and starvation on the $^{125}$I-EGF – EGFR interaction in cultured A) A431, B) U343, C) SKOV3 and D) SKBR3 cells. The interaction was monitored in cells treated with complete medium (red), serum free medium (blue), 1 µM gefitinib (green) and 1 µM gefitinib in serum free medium (black). The affinity of the EGF-EGFR interaction in A431 and SKOV3 increased in the presence of gefitinib and the effect was boosted upon starvation in A431.

The shape of the $^{125}$I-EGF – EGFR binding curves indicated that the interaction was heterogeneous and consisted of a combination of contributing com-
ponents, which was affirmed using kinetic models. A 1:1 model (describing one type of Ligand binding to one type of Target) fitted the data poorly, contrary to the 1:2 model (representing two sets of Target on the cell surface). This showed that the obtained binding curves were the result of two contributing interactions: one fast on – fast off interaction and one higher affinity interaction which had a slower association rate and much slower dissociation rate. Such a heterogeneous behavior was particularly clear in A431, U343 and SKOV3 cells.

The overall apparent affinity of the $^{125}$I-EGF – EGFR interaction was measured by titration of $^{125}$I-EGF, where $K_D$ was estimated as the concentration corresponding to 50 % of the receptors bound (i.e. from which half of the maximum signal could be obtained at equilibrium). The apparent affinity varied greatly between the cell lines, ranging from $K_D \approx 200$ pM in SKBR3 cells to $K_D \approx 8$ nM in A431 cells.

Ligand independent EGFR dimers formed in HER2-rich SKOV3 cells and by exposure of gefitinib

The initial $^{125}$I-EGF – EGFR binding measurements showed signs of two interactions. An early hypothesis was that these corresponded to EGF interacting with either monomeric or dimeric EGFR. EGFR dimers are known to form upon EGF binding and the two observed interactions could have been the result of EGF dissociating from the monomeric and dimeric form. Whether EGFR dimers existed pre-formed and ligand independently could not be determined from this data alone. The amount of EGFR monomers and dimers were therefore quantified by immunoblotting, using the cross-linking reagent bis(sulfosuccinimidyl)suberate (BS3). BS3 forms covalent bonds with closely spaced amines, which ensures that EGFR dimers do not separate during cell lysis. The immuno-blotting experiments were conducted using EGFR-rich A431 cells and HER2-rich SKOV3 cells. In order to study ligand independently formed EGFR dimers, results from EGF non-stimulated cells were compared with data from cells incubated with 9 nM EGF for 3.5 h prior to cell lysis. Cells were grown in either complete (with fetal calf serum, FCS) or serum free medium, in the presence or absence of 1 µM gefitinib. The effect of 20 nM pertuzumab was monitored to separate between the impact of EGFR homodimers and EGFR – HER2 heterodimers. Pertuzumab is a monoclonal antibody with the ability to prevent HER2 dimerization.
Results of the immunoblotting are presented in Figure 16. The intensities were quantified using the software Image J to calculate the effect of a certain treatment, according to

\[
EffectValue_X = \frac{Dimerization_X}{Dimerization_{NoX}} = \frac{Dimer_X}{Monomer_X} \cdot \frac{Monomer_{NoX}}{Dimer_{NoX}}
\]

i.e. the effect of a certain treatment X was studied by comparing otherwise identically treated cell lysates within the same gel. Note that it was the dimer:monomer ratio, not the absolute number of EGFR, that was compared.

EGFR dimers were observed in EGF non-stimulated A431 and SKOV3 cells grown under normal conditions, although to a much lower extent in A431 (Fig. 16 A-D, lanes 1 and 2). The dimerization increased 3.9 times in A431 cells when adding EGF, but no effect of EGF was observed in SKOV3 cells where the dimerization was already high to begin with.

Figure 16. Immunoblots depicting EGFR dimerization patterns in A-B) A431 and C-D) SKOV3 cells in the presence and absence of EGF, gefitinib, pertuzumab and fetal calf serum (FCS). BS3 free lysates were used as negative controls. The similar \(\beta\)-actin band confirmed equal protein loading. This is a representative of one of four experiments.
Gefitinib significantly induced dimer formation in A431 (Fig. 16 A-B, lanes 3, 4, 6 and 7), especially in the absence of EGF where the amount of dimers were otherwise low (3.0-3.8 times increase, Table 2). A smaller, but significant (p<0.1) increase was measured in EGF treated SKOV3 cells (1.8-2.2 times, Table 2).

No effect of the HER2 dimerization preventing antibody pertuzumab was observed in A431 cells. In HER2-rich SKOV3 cells, however, the antibody caused a decrease of the EGFR dimerization by 40-60 %. This lead to the conclusion that a large fraction of the EGFR dimers in SKOV3 were EGFR – HER2 heterodimers.

Table 2. The effect of gefitinib on EGFR dimerization in A431 and SKOV3 cells. The intensities of the monomer and dimer bands were quantified using ImageJ. The table presents EffectValues, which describe the increase of dimer:monomer ratios upon gefitinib exposure by comparing pairs of lysates otherwise treated identically. Gefitinib induced dimerization in A431 cells and in EGF treated SKOV3 cells, irrespective of FCS and pertuzumab. Data are presented as mean±S.E (n = 4). *: treatments affecting dimerization, i.e. with a calculated EffectValue significantly different from 1 (p<0.1).

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<th>A431</th>
<th>Without pertuzumab</th>
<th>With pertuzumab</th>
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<td>With EGF</td>
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<tr>
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<td>3.6±1.0*</td>
<td>1.9±0.1*</td>
</tr>
<tr>
<td>+Gef. –FCS</td>
<td>3.6±0.8*</td>
<td>1.4±0.1*</td>
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<table>
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<tr>
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<th>Without pertuzumab</th>
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<tr>
<td>Effect of:</td>
<td>Without EGF</td>
<td>With EGF</td>
</tr>
<tr>
<td>+Gef. +FCS</td>
<td>1.2±0.1*</td>
<td>2.0±0.1*</td>
</tr>
<tr>
<td>+Gef. –FCS</td>
<td>1.5±0.1*</td>
<td>1.8±0.1*</td>
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Interaction Map as a tool to understand heterogeneity, exemplified by the EGFR system

From the measurements described above, the following conclusions had been drawn:

- The $^{125}$I-EGF – EGFR interaction is heterogeneous, with one fast on – fast off and one slow on – slow off component.
- The interaction kinetics varies between cell lines, with apparent $K_D$ values ranging from 0.2 – 8 nM.
- Gefitinib can increase the affinity in A431 and SKOV3 cells.
- Gefitinib induces dimerization, most evidently observed in EGF non-stimulated A431 cells where the amount of dimers is normally low.
- Pertuzumab, designed to prevent HER2 dimerization, reduces the overall EGFR dimerization in SKOV3 cells but not significantly in A431 cells.
- EGFR dimers in SKOV3 are often EGFR – HER2 heterodimers.
The Interaction Map tool was applied on real-time $^{125}$I-EGF – EGFR interaction data from LigandTracer measurements performed in various cell lines and growth conditions. A few of these are presented in Figure 17.

At normal conditions two interactions were observed in A431 and SKOV3 cells, represented by the map peaks A1/A2 and D1/D2, respectively (Fig. 17 A and D). The main difference between the two interactions was the stability, i.e. the dissociation rate constant $k_d$. The contribution of the right-hand peak to the measured binding curve was lower in SKOV3 cells (D1) than in A431 cells (A1).

Exposure to gefitinib clearly affected the $^{125}$I-EGF – EGFR interaction, but the effect varied between the two cell lines. In A431 cells another peak appeared (B3), corresponding to an interaction with approximately the same stability as B2, but with a higher association rate. This additional peak was absent in SKOV3 cells. Instead, the higher affinity interaction (D2) shifted to an even more stable state (E2) and the contribution of the less stable interaction (E1) was reduced. In other words, gefitinib increased the overall affinity of the $^{125}$I-EGF – EGFR interaction in both cell lines, but in different ways.

**Figure 17.** Interaction Maps representing the binding of $^{125}$I-EGF to A431 (A-C) and SKOV3 (D-F) cells in complete medium (A and D) or gefitinib in serum free medium – in the absence (B and E) or presence (C and F) of the HER2 dimerization preventing antibody pertuzumab. EGF interacts with cells in at least two manners, with one fast on – fast off interaction observed in the same position (i.e. with same kinetic properties) in all six maps (A1, B1, C1, D1, E1 and F1). Addition of gefitinib shifted the overall measured interaction to a more stable state, observed as an additional high affinity interaction in A431 (B3 and C3) and a transformation of the more stable interaction (D2) toward an even lower log($k_d$) in SKOV3 (E3). Pertuzumab partly reduced this effect in SKOV3 cells (F).
Addition of pertuzumab had no clear effect on the interaction pattern in A431 cells in any culturing conditions, here represented by gefitinib in serum free medium (Fig. 17 C). Pertuzumab reduced much of the effect caused by gefitinib in SKOV3, with the more stable interaction (F2) being somewhere in between D2 and E2.

The less stable interaction (A1, B1, C1, D1, E1 and F1) remained on the same position regardless of gefitinib, serum or pertuzumab treatment. This peak was hypothesized to represent the interaction between EGF and EGFR monomers, suggesting that the more stable peak (A2 and D2) corresponded to EGF interacting with dimeric EGFR. The relatively low HER2 expression in A431 cells (Table 1, p. 25) makes HER2 a less likely dimerization partner, leading to the conclusion that A2 represented the interaction with EGFR homodimers. The low impact of pertuzumab on the EGF – EGFR interaction in A431 further strengthened this idea. In contrast, the high HER2 expression in SKOV3 cells likely shifted the EGFR dimer population to a state with mainly EGFR – HER2 heterodimers, also visible by the cells’ sensitivity to pertuzumab in regards to kinetics and amount of dimers (Fig. 18 A). There seemed to be some variation in interaction kinetics between EGFR homodimers and heterodimers, considering the different positions of A2 and D2.

Gefitinib increased dimerization in A431, as observed by immunoblotting. However, the gefitinib-induced dimers in A431 appeared as a new sub-population, with a higher association rate (B3). Gefitinib was able to transform the whole dimer population to a more stable state in SKOV3 (E2). This suggested that the gefitinib-induced homodimers behaved differently than the induced EGFR – HER2 dimers (Fig. 18 B). The concentration of gefitinib used in these experiments may have been too low to affect all dimers in A431 cells, with their high EGFR expression, but enough on the lower EGFR expressing HER2.
Discussion

The results in Paper IV and V showed that the EGF – EGFR interaction is more complex than what is generally discussed or taken into consideration, but the impact of this heterogeneous behavior is yet to be determined. The large variations in affinity and kinetic properties between the cell lines and treatments came somewhat as a surprise. Ligand – Target interactions are often considered relatively independent of model system and data from one
cell line are used to estimate effects in other cell lines. As observed, these kinds of assumptions about cell line independency should be made with care.

It was concluded that the differences of interaction kinetics in cell lines and upon exposure of gefitinib could be associated with EGFR:HER2 ratios and induction of dimers, but these conclusions do not explain why an impact of gefitinib was observed in only two of the four cell lines. U343 is the only one of the four cell lines that is insensitive to gefitinib regarding cell growth [71, 79], which may explain the low impact of gefitinib on the EGF – EGFR interaction. However, this does not explain why SKBR3, being the most gefitinib sensitive of the tested cell lines regarding growth [79], was unaffected by gefitinib as well. It is possible that the already strong binding of EGF to normally treated SKBR3 cells made it difficult to detect any further increase in affinity. The obvious alternative to the cell lines used in this work would be transfected cells. Transformation processes require more time and effort, but would have made the interpretation of data more straightforward.

These papers presented examples of how a combination of established assays can provide new perspectives for understanding intrinsic biological interactions. Interaction Map was further applied as a complementary tool, to strengthen some of the formed hypothesis. Data from conventional methods, such as immunoblotting, made it possible to decipher the map peaks and relate them to EGFR structures. From these observations some general conclusions about Interaction Map were drawn. Once the identity of the peaks are confirmed, Interaction Map can likely be used on its own for understanding heterogeneity in e.g. different culturing environments, reducing the need for more time-consuming manual methods. In addition, applying Interaction Map in an initial stage of a study can be a mean to form an early hypothesis of the nature of an interaction. Although the identities of the peaks may still be unknown, much knowledge can be obtained by the number of peaks alone and how they are positioned in the $k_a/k_d$ space.

Paper VI

Aim

The aim of paper VI was to investigate the EGFR system further. This included the study of dimer formation as well as internalization, recycling and degradation of EGF and EGFR. The three cell lines that were investigated, A431, U343 and SKOV3, cover an interesting span of EGFR and HER2 ratios. By applying a combination of methods and introducing perturbations, such as temperature changes, some of these processes could be separated. The effects of the tyrosine kinase inhibitors gefitinib, lapatinib, AG1478 and erlotinib on the EGF – EGFR interaction were monitored to provide an over-
all idea of how they are associated with affinity alteration and induction of dimers.

Results

Real-time measurements at 37 °C illustrated internalization and ligand processing

The interaction between $^{125}$I-EGF and EGFR was monitored in A431 (Fig. 19 A-B), U343 (Fig. 19 C-D) and SKOV3 cells (Fig. 19 E-F), inside a humidified incubator at 37 °C (grey curves) and at room temperature (black curves) with an incubation time of either four hours (solid line) or two hours followed by a two hour retention measurement (dotted line). The measurements were performed in LigandTracer in complete medium (Fig. 19 A, C, E) or in cells pre-incubated with one of the four TKIs gefitinib, lapatinib, AG1478 and erlotinib. The TKIs affected the interaction in similar ways and are here represented by data from the gefitinib measurement (Fig. 19 B, D, F).

In A431 and U343 cells the signal visibly started to decrease after approximately 40 minutes at 37 °C (Fig. 19 A and C). This likely depicted the excretion of $^{125}$I caused by internalization of the $^{125}$I-EGF – EGFR complex and subsequent degradation within the cells. Such a signal decrease was not observed in SKOV3 cells, indicating that one or several of the processes that eventually result in nuclide excretion were considerably slower.

The signal drop at 37 °C was clearly reduced in A431 and U343 cells treated with any of the four TKIs (Fig. 19 B and D), proposing that internalization rate and/or intracellular degradation were affected by the TKIs.
Figure 19. Real-time LigandTracer measurements of the binding of $^{125}$I-EGF to A-B) A431, C-D) U343 and E-F) SKOV3 cells at room temperature (black curves) or at 37° C (grey curves). Incubation was followed for either four hours (solid line) or two hours followed by a two hour retention measurement (dotted line – retention measurement start indicated by a mark). Measurements were conducted in normally treated cells (A, C, E) or in cells pre-incubated with 1 µM gefitinib, lapatinib, AG1478 or erlotinib, here represented by measurements with gefitinib as the effects were similar (B, D, F).

Acid-wash measurements showed a lower degree of internalization upon TKI treatment

Information about the degree of internalization of a radiolabeled molecule can be obtained by stripping off surface proteins from cells with acid and then disrupt cell structure with a strong base. This was done in A431, U343 and SKOV3 cells on ice, at 7° C inside a cold room, at room temperature and at 37° C inside an incubator (Fig. 20). Cells were un-treated (black, dashed line) or pre-treated with 1 µM gefitinib (light grey, solid line), lapatinib (grey, solid line), AG1478 (grey, dashed line) or erlotinib (black, solid line).
Error bars were calculated as distribution of triplicates, i.e. \( \frac{\text{max value} - \text{min value}}{2} \).

**Figure 20.** Degree of internalization, i.e. internalized \({}^{125}\text{I}\) activity as the percentage of total cell-associated activity in cultured A-B) A431, C-D) U343 and E-F) SKOV3 cells, normally treated in complete medium (black, dashed line) or pre-treated with 1 µM of gefitinib (light grey, solid line), lapatinib (grey, solid line), AG1478 (grey, dashed line) or erlotinib (black, solid line). Error bars indicate distribution of triplicates, \( \frac{\text{maximum value} - \text{minimum value}}{2} \). Degree of internalization was similar at room temperature (A, C, D) and at 37\(^\circ\) C (B, D, F). All TKIs decreased internalization.

Degree of internalization was just as high as, or sometimes higher, at room temperature than at 37\(^\circ\) C for all three cell lines. In SKOV3 the internaliza-
tion was approximately the same as in A431 and U343 cells, despite the low excretion rate observed in the LigandTracer measurements (Fig. 19).

All four TKIs clearly reduced the degree of internalization, although to different extents. Erlotinib had a lesser impact on internalization in all three cell lines, which was also observed for AG1478 in A431 and SKOV3, and lapatinib in U343.

Internalized $^{125}$I-EGF on ice or at 7° C was between 2 and 5 % of the total cell associated $^{125}$I-EGF.

**The impact of TKIs on the $^{125}$I-EGF – EGFR interaction kinetics**

Real-time measurements were conducted at room temperature and at 7° C to monitor the uptake and retention of $^{125}$I-EGF in un-treated (black, dotted line) or gefitinib (light grey, solid line), lapatinib (grey, solid line), AG1478 (grey, dotted line) or erlotinib (black, solid line) treated A431, U343 and SKOV3 cells (Fig. 21), to study the effects of TKIs as well as any differences in interaction kinetics between room temperature and a cold environment. The interaction was characterized in LigandTracer using a step-wise increase of the $^{125}$I-EGF concentration.

No clear differences were observed between the two temperatures. The fact that much EGF was internalized at room temperature (see acid-wash measurements above), but not at 7° C, proposed that there was some recycling processes occurring at room temperature and that these, as well as internalization, was faster than the dissociation. A slow event, such as the dissociation, will be the time-limiting step, making any faster processes difficult or impossible to detect.

Gefitinib, AG1478 and erlotinib increased the affinity of the $^{125}$I-EGF – EGFR interaction in A431 and SKOV3 cells, observed as a slower dissociation rate and/or less signal increase upon addition of more EGF. In contrast, lapatinib made the fast on – fast off contribution of the interaction more abundant in all three cell lines.

The interaction data from room temperature measurements were analyzed with Interaction Map (Fig. 22). As no or little difference was observed between the cell lines, only one (SKOV3) is presented here. The effects of gefitinib, AG1478 and erlotinib were highly similar and are represented by the erlotinib treatment. The degree of contribution was calculated by integration of the map peaks.

The heterogeneity of the $^{125}$I-EGF – EGFR interaction was observed as two major peaks in the maps, with distinctly different dissociation rate constants $k_d$. The indications of an affinity increase caused by gefitinib, AG1478 and erlotinib were explained by the increase in contribution of the more stable component, from 65 % (A1) to 88% (C1). Lapatinib had an opposite effect, by shifting the interaction towards the less stable contributing component (B2), which went from representing 32 % of the measured curve to 67 %.
Figure 21. Binding curves representing the uptake and retention of $^{125}$I-EGF to A-B) A431, C-D) U343 and E-F) SKOV3 cells, either un-treated (black, dotted line) or exposed to gefitinib (light grey, solid line), lapatinib (grey, solid line), AG1478 (grey, dotted line) or erlotinib (black, solid line). The interaction became stronger in gefitinib, AG1478 or erlotinib treated A431 and SKOV3 cells, observed as a slower dissociation rate and less signal increase upon addition of more $^{125}$I-EGF. Lapatinib increased the contribution of the fast on – fast off component of the interaction in all three cell lines.

The two peaks likely represent EGF binding to EGFR monomers (A2, B2, C2) and dimers (A1, B1, C1), as discussed in Paper V. The TKIs can affect the dimer:monomer ratio [70, 72-75], which was clearly visible in the Interaction Maps. This proposes that Interaction Map can be used as a tool to explain structural relationships without the need of more time-consuming methods such as immunoblotting.
Figure 22. The heterogeneous $^{125}$I-EGF – EGFR interaction in A) normally, B) lapatinib or C) erlotinib treated SKOV3 cells at room temperature, as displayed in Interaction Maps. Two peaks were observed, corresponding to interactions with similar association rates ($k_a$) but with different dissociation rates ($k_d$), separated by more than two orders of magnitude. Exposure of lapatinib increased the contribution of the less stable interaction, from 32 % (A2) to 67 % (B2). Upon gefitinib, AG1478 and erlotinib treatment, here represented by erlotinib (C), the interaction shifted to the more stable state.

Complementing methods can propose a mechanism of EGFR dimerization and internalization

By the combination of measurements presented in Paper VI, a hypothesis was formed describing the complex pattern of interaction kinetics, internalization, recycling, degradation and excretion of EGF and EGFR (Fig. 23). When EGF interacts with EGFR, the receptor dimerizes with other active EGFR (in EGFR-rich cells such as A431 and U343) or HER2 (in SKOV3).
The dissociation of EGF from EGFR dimers is considerably slower than from monomers. At room temperature and at 37° C the EGF-EGFR complex is eventually internalized. The internalized complex can be recycled or degraded. Degradation mainly occurs at 37° C and then most visibly for EGFR homodimers.

Gefitinib, AG1478 and erlotinib induce formation of dimers. These dimers have a slower internalization rate and are possibly also degraded to a lesser extent.

**Figure 23.** The proposed mechanism of interaction kinetics, internalization, recycling, degradation and excretion of EGF and EGFR in different cell lines, treatments and temperatures. The interaction of EGF with EGFR monomers (black square, dashed line) causes the conformation shift necessary to form dimers, typically homodimers (black square, solid line) in EGFR-rich A431 and U343 cells and EGFR – HER2 heterodimers (black square, dotted line) in SKOV3 where the HER2 expression is high. Exposure of gefitinib, AG1478 or erlotinib increases the dimerization, but creates dimers that seem to be catalytically inactive (grey squares). At room temperature and 37° C the dimers can internalize, but excretion due to degradation requires temperatures around 37° C and is then still low for EGFR – HER2 heterodimers. Gefitinib, lapatinib, AG1478 and erlotinib reduce dimerization.

**Discussion**

Increasing the temperature to 37° C makes any interaction data much more complicated to interpret, but the use of physiological temperatures will even-
tually be necessary to get the complete picture of a protein system. The work presented in Paper VI describes how a set of complementary methods in combination with perturbations can be used to grasp the details of an interaction and the destiny of its components. This added another piece to the intricate EGFR puzzle.

With the high costs involved in drug development every tool that can reduce development time is of interest. Much effort is often spent on structural analysis, including time-consuming crystallography processes. In this paper we show that the use of Interaction Maps can describe some of the structural effects involved in TKI exposure. The use of Interaction Map as a tool to choose suitable drug candidates resulted in a patent application [80].
Ongoing studies

This thesis describes the development of analysis tools for investigation of specificity, receptor quantities, interaction kinetics, heterogeneity and ligand degradation, among others. The tool kit was used to obtain new information on the EGFR system, which is a relevant target for anti-cancer research. Just as this PhD project, the ongoing studies focus on both the development of methods and the biology to which they are applied.

In Paper III we challenged Interaction Map through quantification, exploring how well the tool adapts to variation in e.g. ratios of Targets [81]. Similar measurements of precision and robustness are ongoing and will continue in the future. Historical data sets are analyzed to further compare the accuracy of the estimation of $k_d$ and $k_d$ of each contributing interaction. The smallest difference in kinetic properties that Interaction Map can separate is currently investigated by the use of simulations. Optimizing peak separation will likely require a higher pixel resolution as well as a reduction of noise and error of the real-time detection instruments.

More studies are planned to understand how the results of Interaction Map will depend on signal-to-noise ratio, ligand concentration, data resolution, choice of algorithm, etc., and if it is possible to correct for assay specific effects such as Ligand depletion and Ligand diffusion limitations. Such analysis will likely be performed using data from different instrument platforms, with e.g. immobilized purified protein systems to create artificial heterogeneity.

Our group had extensive discussions when preparing Paper III. It is clear that the principle of Interaction Map, as well as the data it generates, is far from easy to comprehend. As developers it is crucial that we try to improve the presentation of the tool in order to simplify the understanding for newcomers, and to ensure that it is accessible even for biologist not too familiar with mathematical fitting algorithms. Details, such as nomenclature, may therefore need further polishing.

Being partly associated with the academic world it comes naturally to participate in various research projects and to exchange knowledge with coworkers from the University. The findings in Paper I and II have been valuable not only within our own research group, but also for several of the groups that we come into contact with. We keep on emphasizing the importance of investigating interactions in real-time and continuously support our colleagues with assay design and kinetic evaluations.
In Nilvebrandt et al., the interactions between CD44v6 binding antibody fragments and their target were detected in LigandTracer and analyzed using Interaction Map [82]. The study has continued with a thorough investigation of how labeling, hosting cell line and dimer forms affect the interaction kinetics of one of the antibody fragments – as measured on cells and with xenografts in mice.
Future Perspectives

As an industry sponsored PhD student associated with both a university (Uppsala University) and a company (Ridgeview Instruments AB), my research has always had two approaches – a scientific and a commercial. Although new scientific findings has been the main goal throughout the project, my research required much support from the company, which in turn would not have been possible (economically speaking) if the company could not have benefitted from it. In order to speculate on what will become of my research one must first accept the duality of this project and understand that there will be scientific and commercial aspects of it in the future as well.

From a scientific point of view, I believe that Interaction Map has the potential to answer some of the questions associated with complex environments such as cells. This PhD project focused on the EGF – EGFR interaction, but it has also been employed in the analysis of several other surface receptors, including CD44v6 [82] and Neuropilin-1 [83]. The cell is full of advanced receptor systems that are yet to be thoroughly understood and Interaction Map may be a valuable tool for grasping the heterogeneity aspect of these, to provide explanations of e.g. differences between cell lines, treatments and patients.

To the best of my knowledge, real-time interaction analysis has never before been implemented for the understanding of cellular processes present at physiological temperatures. One initial goal of Paper VI was to create a tool in MATLAB which could fit the generated data and estimate kinetic rate constants of e.g. internalization and degradation. This required a mathematical model that could describe the simultaneously occurring events. It was unfortunately found that such a model could not be simplified enough to make a good estimation of all kinetic constants, when using the data from the limited number of measurements that had been conducted. Developing tools for interaction analysis at 37°C may continue in the future. If a fitting tool is to be created it will require extensive planning to obtain all necessary data, in which the experiences from Paper VI will be highly valuable.

Commercially, Interaction Map is promising. During my PhD studies, two new patents have been filed describing applications of it [80, 84] and a sister company to Ridgeview Instruments AB has been formed called Ridgeview Diagnostics AB (RDAB). Interaction Map is currently available from RDAB as a pay-per-calculation tool for a few initial customers, using a mail service in combination with the evaluation software TraceDrawer.
Several pharmaceutical companies have already shown great interest in using Interaction Map to better understand their interaction data.

In the paper written by Gedda et. al., Interaction Map was used to analyze the interaction of the HER2 binding DAKO antibody with breast cancer patient tissue samples. It was found that results from Interaction Map analysis reflected the immunohistochemistry (IHC) HER2 scoring of the samples well [85]. IHC, the golden standard for investigation of tissue sections, provides spatial resolution, but relies on operator dependent, multi-layered, endpoint measurements which increase the risk of inaccurate data. This has resulted in a general call for standardization of IHC the last couple of years [86]. Interaction Map applied on operator independent real-time data may be such a standardized platform. One may further speculate on a future integration of Interaction Map into the field of personalized medicine, a field which is especially important in cancer treatment due to the intrinsic heterogeneity of the disease. Investigations will continue to analyze potential connections between antibody – antigen interaction kinetics and how patients respond to certain treatments.
Populärvetenskaplig Sammanfattning


Ett problem med ovannämnda strategi är att den mest detaljrika informationen om hur läkemedlet binder till proteinet samlas i det tidiga, artificiella, systemet, men att denna information inte alltid återspeglar verkligheten väl. När man sedan ger läkemedlet till djur och människor kanske man inte får den biologiska effekt som man hade tänkt sig och det är då svårt att spåra varför, eftersom det kan pågå så mycket annat i en levande organism som inte sker i de artificiella systemen. En första lösning på ett sådant problem är att skapa en testmiljö som bättre motsvarar den komplexa verklighet som finns inne i ett djur eller människa.


Ett tidigt stadium i arbetsprocessen var att först acceptera att det kanske inte bara är en sorts interaktion som sker i cellen, utan flera. Binder min molekyl till protein X kanske det också är troligt att den gör det till dess kusiner Y och Z. Målet var sedan att hitta metoder för att skilja på dessa interaktioner. På så vis skulle en mer fördjupad kunskap kunna erhållas om hur proteiner beter sig i deras rätta miljö.
Mycket av arbetet i denna avhandling beskriver bindningsstudier utförda i real-tid. Sådana real-tidsstudier ger information inte bara om hur starkt molekyler binder till varandra, utan även hur snabbt de börjar interagera och hur länge de fortsätter göra det. Det här är ett sätt att mäta som har varit möjligt de senaste decennierna men ännu inte helt anammats eftersom det ofta kräver dyra instrument och genererar relativt svårtolkade data. Fördelen med att studera bindningar över tid är dock många. Vid t ex läkemedelsutveckling är det av högsta vikt att man vet att molekylen hinner binda till dess mål innan det sköljs ut ur kroppen, och att molekylen förblir bunden under tillräckligt lång tid för att hinna ha en biologisk effekt.

I arbetet inför Artikel I användes kunskap om hur bindningar förändras över tiden för att optimera en klassisk biologisk mätmetod. Tidsperspektivet applicerades först teoretiskt genom att göra simuleringar på datorn. Detta generade idéer om hur man bör tänka när man designar sitt experiment, med avseende på molekylkoncentrationer och mättider. Idéerna granskades sedan genom att återskapa de simulerade bindningarna i riktiga biologiska system, där samma slutsatser om koncentrationer och mättider kunde dras. Den samlade kunskapen om interaktioners tidsberoende var mycket viktig för de fortsatta studierna i detta doktorandprojekt.

Artikel II beskriver likt Artikel I hur automatiserade real-tidsmätningar kan användas för att förbättra en befintlig manuell metod, i detta fall en metod för att studera receptorantal på celler. Genom att göra matematiska anpassningar av real-tidsdata erhålls liknande resultat som när receptorantalet mättas manuellt, men med lägre åtgång av reagens och arbetstid. Denna metod, kallad KEX, användes senare i Artikel V.


Till Artikel IV studerades hur EGF binder till EGFR på fyra olika typer av cancerceller. En stor variation mellan celltyperna uppmättes. Dessutom syntes spår av två sorts bindningshastigheter, något som antydde att EGF kan binda till olika EGFR-varianter på celler. I Artikel V visades det att hastigheterna som uppmättes var EGF som band till antingen vanliga EGFR
eller till så kallade EGFR-dimerer, d v s två EGFR som sitter ihop med
varandra. EGF förblev bundet till EGFR-dimerer längre än till ensamma
EGFR.

Till Artiklarna IV och V studerades även hur EGF interagerar med EGFR
i närvaro av anti-cancer-läkemedlet gefitinib. Gefitinib binder till den del av
EGFR som sitter på insidan av cellerna, men verkade ändå kunna påverka
EGF-EGFR-interaktionen som sker på utsidan av cellen, på ett sätt som
gjorde att EGF ville sitta kvar på EGFR längre. Det visade sig att gefitinib
genererar fler EGFR-dimerer. Gefitinib har en tumörhämmande effekt hos
bara en liten andel av de patienter som behandlas med det. Att få en mer
detaljerad förståelse för hur EGF binder till EGFR är betydelsefullt både för
att förstå hur man ska skapa läkemedel och för att kunna välja ut rätt patien-
ter för en viss behandling.

Artikel V beskriver även hur Interaction Map kan användas för att ytterli-
gare förstå den komplexitet som finns i celler. Det visade sig att resultat från
Interaction Map stämde väl överens med de resultat som hade samlats med
en kombination av vedertagna metoder. Interaction Map skulle eventuellt
kunna användas frittstående i framtiden, utan att behöva komplettera med de
etablerade metoderna.

I Artikel VI granskades EGF-EGFR-interaktionen mer djupgående. Istäl-
let för att studera bindningen i rumstemperatur, vilket gjordes i Artikel IV
och V, så utfördes mätningar i bland annat 37° C. Vid kroppstemperatur är
betydligt fler processer i cellen påslagna, något som kan försvåra tolkningen
av experimentella resultat. I artikeln beskrivs hur man med en kombination
av mätmetoder och sätt att störa systemet ändå kan få förståelse för vad som
sker. Bland annat uppmättes hur EGF och EGFR tog sig in i cellen och så
småningom bröts ner. Det här är ett försök att komma ytterligare ett steg
närmare vad som faktiskt sker i våra egna kroppar, för att på så vis bättre
förstå sjukdomsprocesser och hur man ska kunna förhindra dem.

Allt som allt kan man beskriva det här arbetet som en kombination av bio-
logi, teknik, matematik och programmering, med mål att skapa mätmetoder
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine.