

Exploring Time-Resolved Characterization of the Heterogeneity and Dynamics of Ligand-Receptor Interactions on Living Cells

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Abstract: The time-resolved interaction analysis was applied on living cells to extract detailed interaction characteristics of two therapeutic antibodies and natural ligand binding to the same receptor expressed on two different human carcinoma cell lines.

The observed differences in the antibody binding characteristics and heterogeneity could be attributed both to differences in antibodies and cell lines. The stability of antibody binding to EGFR on cells is significantly higher than the binding stability to isolated EGFR. This higher stability can be of fundamental importance as it potentially shifts the drug-target residence time into a domain that is limiting in pharmacokinetics and hence is of importance for *in vivo* drug efficacy.

EGF binding to its receptor was more heterogeneous and it was demonstrated for the first time that time-resolved interaction measurements in combination with Interaction Map analysis could be used to probe the dynamics of a ligand (protein) induced dimerization and/or oligomerization process.

Keywords: Cetuximab, EGF receptor, Interaction Map, kinetics, panitumumab, tracer.

INTRODUCTION

Molecular recognition is a key element in both biology and medicine. For example, many therapeutic antibodies act through binding to a receptor thereby turning off the signalling cascade initiated by the receptor. Other effector functions are coupled to the Fc part of the antibody, such as the antibody-dependent cell-mediated cytotoxicity mechanism for cell killing. Another example is the development of *in vivo* diagnostic agents, wherein a tracer molecule is designed to carry a label to a target structure. Targeting tumor-specific antigens with PET/SPECT-tracer labeled antibodies belongs to this class. Understanding and characterizing protein-receptor interactions with respect to the rate and degree of receptor occupation are therefore crucial in the development of proteins used for therapeutic or diagnostic purposes.

Time-resolved binding assays not only reveal the binding strength (affinity) of the interaction but also the rate in which association and dissociation takes place. While the affinity of the interaction gives the information

on the drug concentration required for blocking a sufficient percentage of targets, the dissociation kinetics is considered of importance to estimate and optimize the drug-target residence time [1, 2]. Time-resolved binding assays have a strong position in biophysics and have become standard practice in the characterization of molecular interactions and development of therapeutic proteins [3-6]. Recently, however, the relevance of the drug-target residence time was questioned because dissociation rate, as measured with biophysical methods, is most often too fast in relation to pharmacokinetic elimination of the drug from blood [2].

Since the majority of diagnostic and therapeutic proteins are intended for use in patients to monitor, reduce or cure malfunction such as cancer, it is highly relevant to investigate the drug-target interaction properties in an experimental setting that resembles the *in vivo* working environment of the drug. With the advent of time-resolved technologies capable of monitoring interactions on living cells, the experiences from biophysics can be transferred to cell-based assays. In this manuscript we describe the usage of LigandTracer, a relatively new method for real-time characterization of labeled proteins binding to their receptors on living adherent cells [7-10]. Binding curves obtained from time-resolved biophysical binding assays are not always straightforward to analyze because of

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interaction heterogeneity [6]. Interactions of a protein with receptors expressed on living cells often become even more complex [7]. Recently, the novel evaluation tool Interaction Map was presented as a method to decompose complex binding curves into the underlying components [11, 12].

Epidermal growth factor (EGF) receptor (EGFR/ErbB1) is a tyrosine kinase that has come to the forefront in drug development due to its overexpression in many malignancies like gliomas, head and neck tumors, or colorectal, pancreatic, prostate, non-small cell lung, cervical and breast cancer [13, 14]. It can be generally stated that the greater the receptor expression in the tumor cells, the worse is the prognosis for the patient. Several studies have proved that EGFR-mediated signals are crucial to cancer progression like neoangiogenesis, metastatic spread and apoptosis inhibition. Moreover, EGFR overexpression is associated with resistance to hormone therapy or chemotherapeutic agents [15]. EGFR is targeted by different natural ligands like EGF and transforming growth factor alpha (TGF- α) [16, 17]. When a ligand binds to the extracellular domain of EGFR, the dimerization of receptor with the neighboring EGFR (homodimerization) or with other members of the ErbB family (heterodimerization) is stimulated which in turn leads to intracellular tyrosine kinase domain activation.

Targeting the EGFR family receptors is common and anti-EGFR substances can be divided into two groups. The first group includes tyrosine kinase inhibitors (TKIs) like gefitinib or erlotinib and the second group consists of monoclonal antibodies (mAb) like cetuximab or panitumumab [18]. Cetuximab and panitumumab interact with the extracellular domain of EGFR where a structural analysis of the cetuximab-EGFR interaction revealed that cetuximab partially blocks the ligand binding region and prevents dimerization [19].

In this report we demonstrate the potential of measuring complex and dynamic interactions using LigandTracer and Interaction Map technologies and illustrate and discuss the relevance of cell based assays and detailed analysis of interaction characteristics in relation to *in vitro* protein-protein interaction kinetics as measured with biophysical techniques and *in vivo* pharmacokinetics. As a model system, two ^{131}I - or ^{125}I -labeled therapeutic antibodies as well as the labeled natural ligand EGF binding to EGFR expressed on two different human cancer cell lines were investigated.

MATERIALS AND METHODS

Cell Lines and Proteins

The cell lines used in this study were the human squamous carcinoma cell line A431 (human skin epithelial cells derived from an epidermal carcinoma of the vulva taken from an 85 year old female; CLR 1555; ATCC, Rockville, Maryland, USA) and the human keratinocyte cell line HaCaT (spontaneously transformed human keratinocytes having the characteristics of basal epidermal keratinocytes; DKFZ, Heidelberg, Germany) [20], both expressing the EGFR receptor in high quantity. EGFR signaling is primarily transduced through the process of internalization, ubiquitination and degradation of the receptor–ligand complex, resulting in the transient down-regulation of EGFR [21]. Cells were cultured in conditions presented previously [9]. The cells were seeded on the whole surface of a Petri dish and left to grow to confluence. On experimental day, cells were scratched off (2/3 of a Petri dish surface) and a sector with cells was left when used in LigandTracer Yellow (^{131}I) or LigandTracer Grey (^{125}I) experiments. Alternatively, cells were seeded on a portion of a Petri dish by placing the dish on a tilted support followed by incubation in a horizontal position after replacing the cell containing medium with fresh medium (^{125}I -labelled ligands only). The proteins used in this study included EGF (Chemicon International, USA; GIBCO, California, USA), cetuximab (Merck, Darmstadt, Germany) and panitumumab (AMGEN, Breda, The Netherlands).

Radiolabelling

For radiolabeling, a total of 2.5 μg of human EGF, 80 μg of cetuximab, or 80 μg of panitumumab was labeled with 10–15 MBq ^{131}I (Institute of Isotopes Co., Ltd., Budapest, Hungary) or ^{125}I (Perkin-Elmer, Waltham, MA, USA) according to the chloramine-T protocol [22]. The labeling reactions were performed with chloramine-T (Sigma, St. Louis, Missouri, USA) and sodium metabisulfite (Aldrich, Stockholm, Sweden). The desired radiolabeled quantity of EGF, cetuximab or panitumumab was purified on a NAP-5 column (GE Healthcare, Waukesha, Wisconsin, USA) equilibrated with PBS (10 mM, pH 7.4, 140 mM NaCl).

Interaction Measurement Assay

Regular time resolved interaction measurements were conducted in duplicate through first incubating the cells (A431 or HaCaT) with 3 nM ^{131}I -labeled protein

during 2 hours, immediately followed by an incubation of the same protein at a 10 nM concentration for another 2 hours. After this two-step monitoring of protein binding, the protein containing incubation medium was replaced with pure cell culture medium and dissociation of bound protein was monitored for two to six hours. This procedure is similar to what has been published previously [23]. These regular interaction measurements were conducted at room temperature (approximately 25°C) in full cell culture medium (Dulbecco's Modified Eagle's medium with high (A431) or low (HaCaT) glucose content; 10% of fetal bovine serum (FBS); 5 ml per a petri dish) in LigandTracer Yellow (Ridgeview Instruments AB, Uppsala, Sweden).

Ligand Exposure Time Assay

To study the effect of ligand exposure time on the interaction kinetics, a single 30 nM protein

concentration was exposed to A431 cells for various times. For ^{125}I -cetuximab, exposure times were 2, 8, 16, 32, or 130 min and for ^{125}I -EGF exposure times were 3, 7.5, 15, 30, or 60 min. EGF binding experiments were duplicated and resulting binding curves were co-evaluated. After exposure to either cetuximab or EGF, dissociation was monitored for 30 min in pure cell culture medium. Kinetics runs were made in full culture medium (Ham's F10, 10% FBS, 1% L-glutamine and 1% penicillin and streptomycin). This exposure time study was performed at room temperature (approximately 22°C) using LigandTracer Grey (Ridgeview Instruments AB, Uppsala, Sweden).

Interaction Map Analysis

All resulting binding traces were subjected to Interaction Map analysis (Ridgeview Diagnostics AB, Uppsala, Sweden), as described previously [8]. In brief, Interaction Map decomposes a binding trace into its

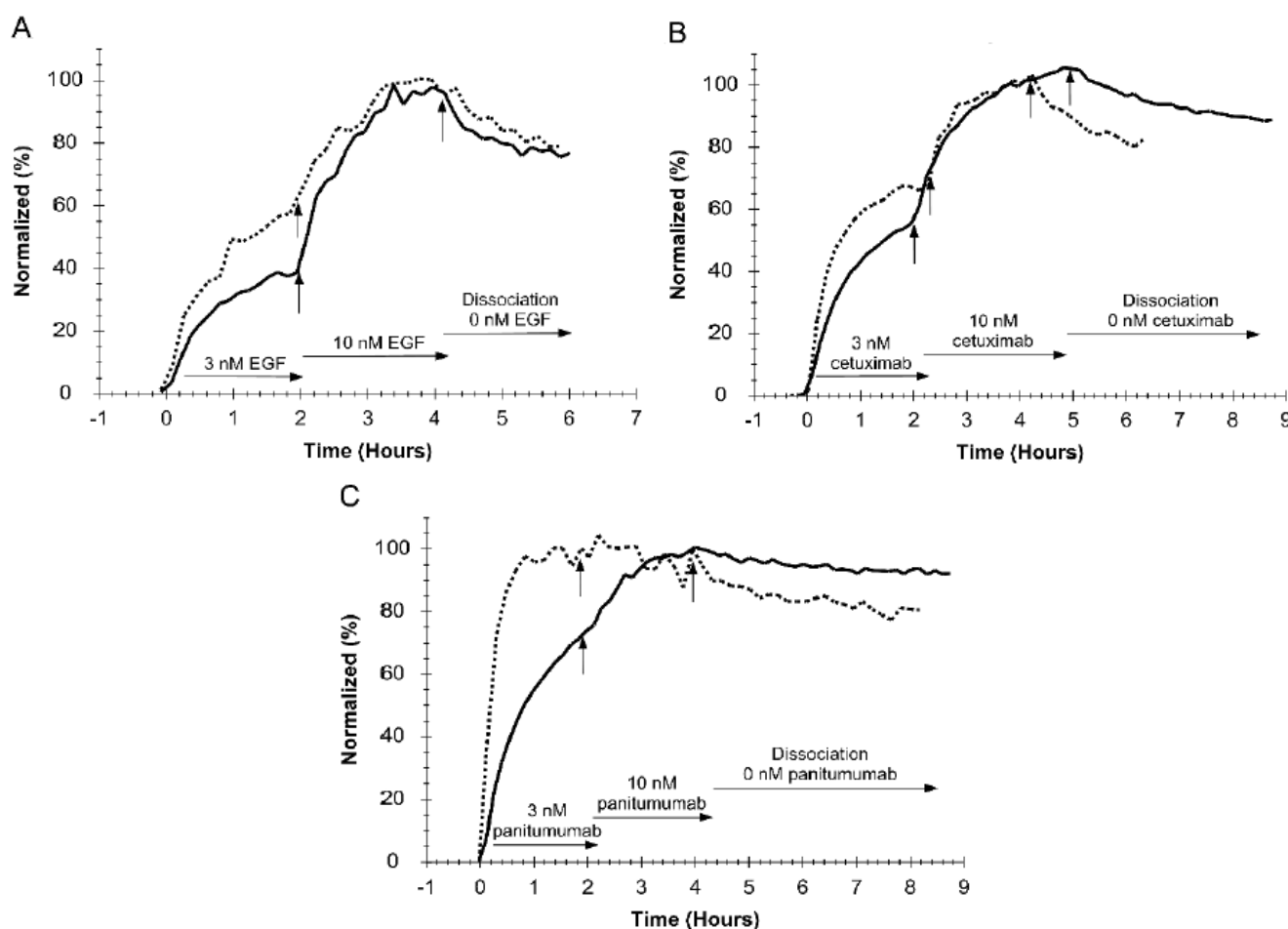


Figure 1: Binding traces from three different radio-iodinated ligands (**A:** EGF, **B:** Cetuximab, **C:** Panitumumab) binding to EGFR on two different human carcinoma cell lines (A431: solid line, HaCaT: dotted line). Each ligand was first incubated at 3 nM concentration during 2 hours, followed by 10 nM incubation for next 2 hours and finally followed by dissociation measurement without ligand for several hours. Duplicates were measured for each ligand and cell line and one representative plot was chosen for each panel.

underlying parallel interaction processes and visualizes the result as a topographic map with log10 (association rate) on the Y-axis, log10 (dissociation rate) on the X-axis, and grayscale or color scale to visualize its relative contribution to the overall interaction process. A single peak means that the binding trace contains data from one binding-like process with interaction parameters determined by the location of the peak, two peaks means that there are two binding-like processes, etc.

RESULTS

Interaction Measurement Assay

The kinetics of ligands (EGF, panitumumab, cetuximab) binding to EGFR on either A431 or HaCaT cells was monitored with the employment of the time resolved interaction measurements. Each ligand was firstly incubated 2 hours at 3 nM concentration followed by addition of a second aliquot of the same ligand to a final concentration of 10 nM and left to interact for another 2 hours. After this two-step incubation, dissociation was followed for several hours with fresh, ligand free medium. All combinations (EGF, panitumumab, cetuximab) with cell lines (A431 and HaCaT cells) produced clear binding curves, as shown in Figure 1. On A431 cells, the interactions of the proteins with EGFR did not reach equilibrium after 2 hours. Binding of the proteins to EGFR on HaCaT cells was faster than binding to EGFR on A431 cells and after two hour incubation the interactions were closer to equilibrium binding levels. Panitumumab was dissociating slower than cetuximab.

Results from Interaction Map analysis of these binding curves are displayed in Figure 2 and extracted kinetic rates and affinity values are given in Table 1. ^{131}I -EGF binding to EGFR resulted in multi-peak interaction maps, indicating that the ^{131}I -EGF-EGFR interaction is of heterogeneous nature. EGFR receptors on HaCaT cells generally displayed higher binding affinity for all proteins than EGFR receptors on A431 cells, in particular for the mAb cetuximab and panitumumab. The mAb interactions with EGFR on A431 cells were primarily characterized by a single affinity peak, where ^{131}I -panitumumab was binding with approximately eight times higher affinity than ^{131}I -cetuximab. Compared to mAb binding to EGFR on A431 cells, the mAb-EGFR interactions on HaCaT cells were more heterogeneous. The dominating contribution (>60% weight in Interaction Map) on HaCaT cells had a high affinity and this affinity was six to nine times higher

than observed for the mAb interactions on A431 cells. The minor contribution (<25% weight in Interaction Map) has a more than 100-fold weaker affinity. This lower affinity is primarily caused by an increased dissociation rate and thus a reduced stability of the binding compared to the high affinity mAb-EGFR interactions.

Ligand Exposure Time Assay

To investigate the heterogeneity of mAb and EGF interactions with EGFR in further detail, experiments were performed with varying exposure times of ^{125}I -cetuximab and ^{125}I -EGF to A431 cells. The concentration of ^{125}I -cetuximab and ^{125}I -EGF was relatively high (30 nM) to provide rapid occupation of binding sites on A431 cells. Interaction Map analysis of ^{125}I -cetuximab binding to A431 receptors and exposure times ranging from 2 min to 130 min resulted in maps (data not shown) that are all similar to the map obtained with the regular interaction experiment as shown in Figure 2. The average affinity obtained from Interaction Map analysis of six ^{125}I -EGF/EGFR on A431 binding assays with association times ranging from 2 to 130 minutes amounted to 216 pM. This value is very similar to the 220 pM affinity that was observed for binding of ^{131}I -cetuximab to EGFR on A431 cells using the regular assay (see Table 1). The individual affinities derived from the 6 assays with varying association times ranged from 82 to 569 pM. This variation is not surprising as for kinetic characterization of interactions it is general advised [25] and common practice [26] to perform experiments with multiple concentrations while the association time variation study was performed with a single ligand concentration and relative short assay times.

The interaction between 30 nM ^{125}I -EGF and EGFR on A431 cells was characterized by a rapid association during the first few minutes followed by a slow increase in binding levels.

Exchanging ^{125}I -EGF containing medium with pure medium resulted in a dissociation pattern where a fraction of the ^{125}I -EGF was released during the first 15 minutes followed by a much slower dissociation afterwards. For the shortest exposure time of 3 minutes, ^{125}I -EGF primarily displayed a rapid association and a binding level indicating that about half of the available binding sites were occupied. With increasing exposure time, more binding sites became occupied and saturation was reached in half an hour. After ^{125}I -EGF exposure, dissociation was measured

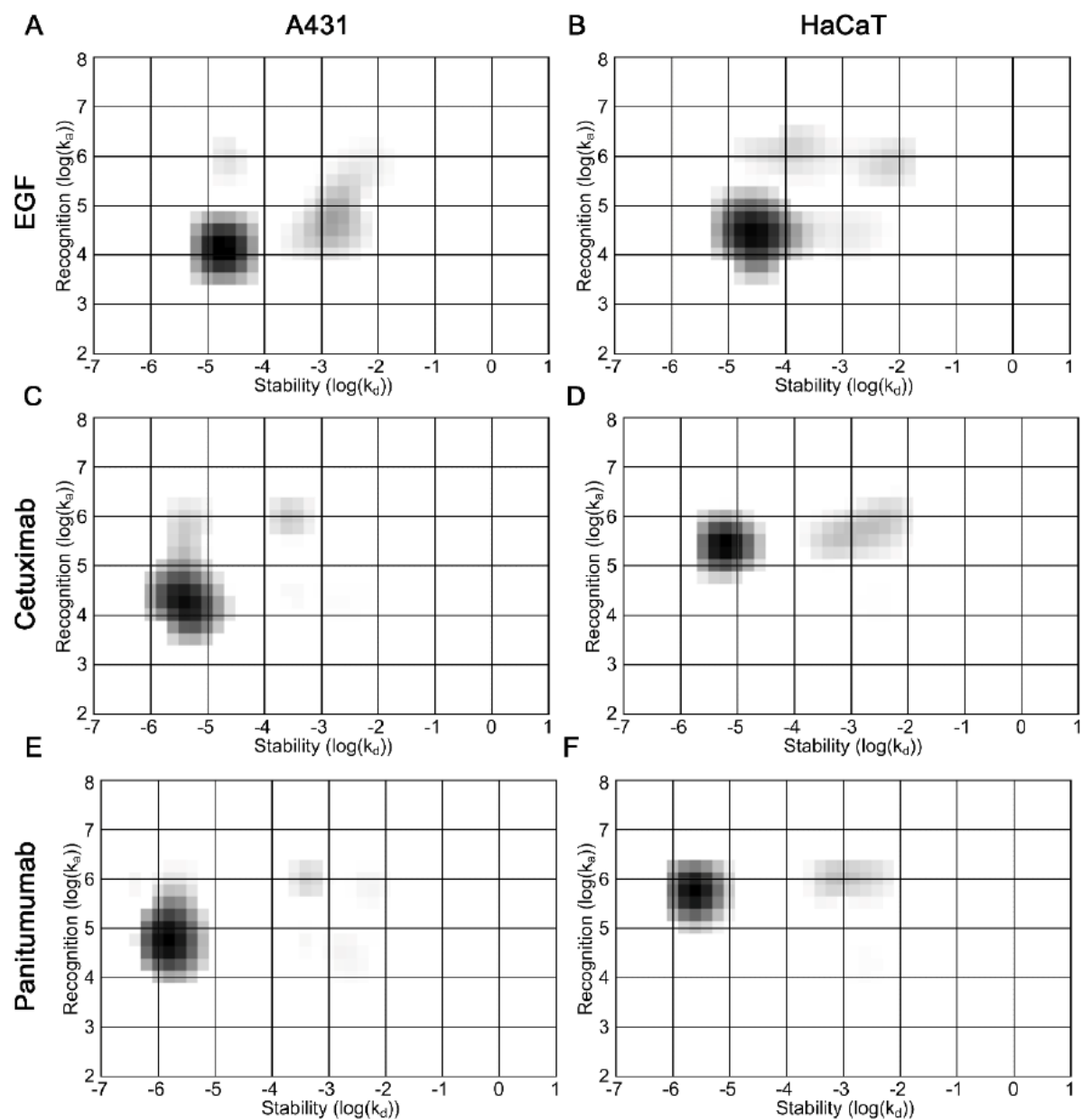


Figure 2: Interaction Maps for ^{131}I -EGF [24], ^{131}I -cetuximab (middle), and ^{131}I -panitumumab (bottom) binding to EGFR on A431 cells (left) or HaCaT cells (right).

Table 1: Interaction Characteristics Extracted from Interaction Map Analysis

^{131}I -labeled ligand	Cell-line	Dominant peak			Secondary peak		
		Position [$\log_{10}k_a$; $\log_{10}k_d$]	KD (pM)	Weight (%)	Position [$\log_{10}k_a$; $\log_{10}k_d$]	KD (pM)	Weight (%)
EGF	A431	[4.1; -4.7]	1600	57	[4.7; -2.8]	33000	21
EGF	HaCaT	[4.4; -4.5]	1100	67	^a	^a	^a
cetuximab	A431	[4.3; -5.4]	220	70	[6.0; -3.6]	290	5
cetuximab	HaCaT	[5.4; -5.2]	25	61	[5.7; -2.8]	3200	22
panitumumab	A431	[4.8; -5.8]	27	75	[6.0; -3.4]	430	4
panitumumab	HaCaT	[5.7; -5.6]	4.6	72	[6.0; -2.9]	1200	14

^aThe ^{131}I -EGF-EGFR interaction on HaCaT cells displayed 2 secondary peaks, each with a weight contributing of 7%.

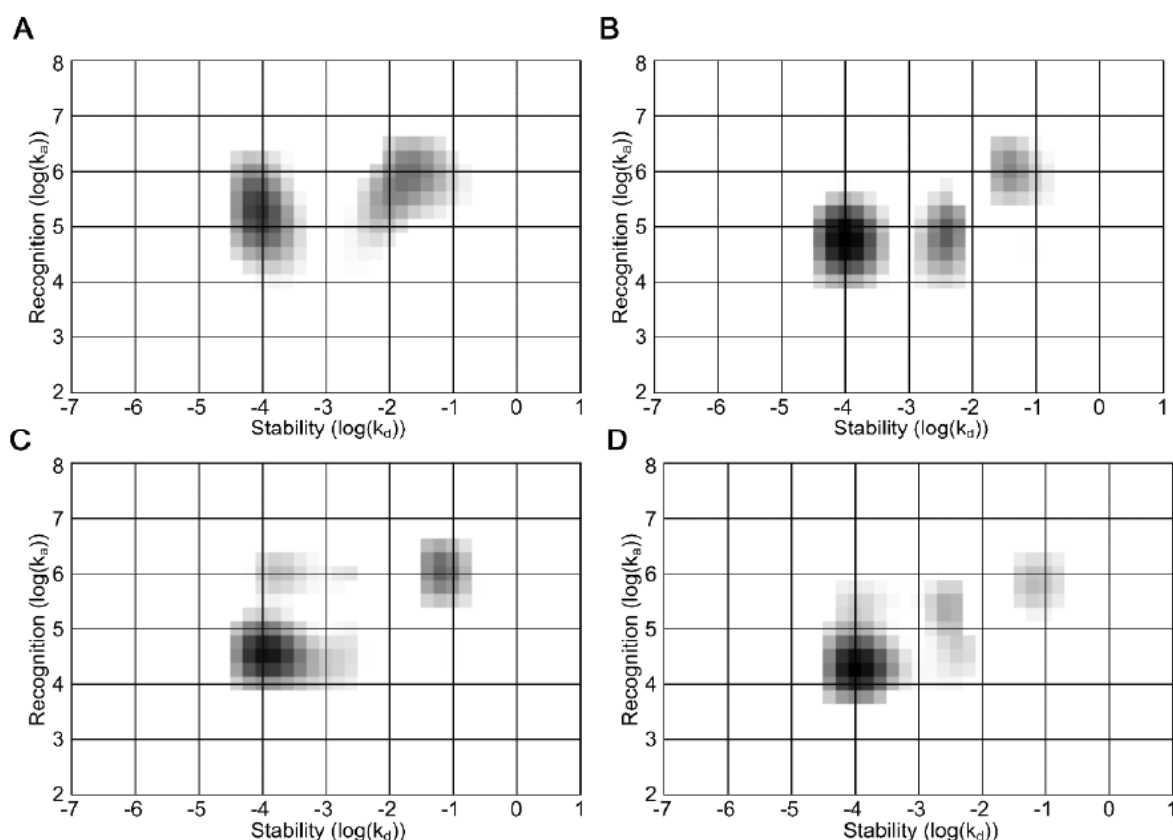


Figure 3: Interaction Maps from 30 nM ^{125}I -EGF binding to EGFR on A431 cells with exposure times of **A:** 3 min **B:** 7.5 min, **C:** 15 min and **D:** 30 min. Dissociation was monitored for 30 minutes. Interaction Map analysis was performed on two binding curves that were obtained from duplicated experiments.

and for an exposure time of three minutes almost 50% EGF was released from its receptor within 15 minutes. With increasing exposure times, this release during the first 15 minutes of dissociation gradually decreased to 34% after 7.5 minutes, 23% after 15 minutes, and less than 20% for a one hour ^{125}I -EGF exposure.

Interaction Map analysis for ^{125}I -EGF EGFR binding with exposure times of 3, 7.5, 15 and 30 minutes are shown in Figure 3. From this figure, it can be seen that the interaction that is characterized by a high stability (left on x-axes) became more dominant and less heterogeneous, i.e., covering less area in the map when the ^{125}I -EGF exposure times increased. The weight of the high stability peak increased from roughly 50% after 3 min exposure to 60% at 7.5 minutes and >70% after 15 and 30 minutes exposure. The affinities from this dominating stable interaction with exposure times from 3 to 30 minutes ranged from 0.6 to 5.5 nM with an average of 3 nM.

The small peak with an association rate of $10^6 \text{ M}^{-1}\text{s}^{-1}$ and a dissociation rate of 10^{-1} s^{-1} , as visible in Figure 3B, 3C, and 3D, is sometimes seen when analyzing LigandTracer cell based data with Interaction

Map. This peak corresponds to a small and quick signal change when radioactive material is added to or removed from the Petri dish and most likely reflects a difference in background signal between cell covered and cell-free areas of the Petri dish rather than a biological binding event.

DISCUSSION

Time-resolved interaction analysis is powerful for determining how molecules interact so that not only the affinity or binding strength but also kinetic properties can be revealed like the association rate constant that represents how well proteins recognize each other and the dissociation rate constant that represents the stability of the interaction. This report describes the detailed characterization of the molecular interactions of three ligands binding to the same receptor as expressed on two different cell lines. The obtained results revealed several similarities and differences between both molecules and cells. Compared to binding to EGFR on A431 cells, binding of all three proteins to EGFR on HaCaT cells consistently resulted in higher association rates leading to approximately 10 times higher affinity (see Figure 1 and Table 1). This

finding confirmed the results of recent studies that the same protein binding to the same target on different cells can produce very different results [7, 12, 27]. Also antibody binding to the EGFR varied as on both cell lines binding with ^{131}I -panitumumab resulted in approximately 10 times higher average affinity for the dominant peak than binding with ^{131}I -cetuximab.

Today's therapeutic proteins often present a technical challenge in that their affinity to the target protein is very high, often at the level of single digit picomolar K_D . In this report, panitumumab and cetuximab interactions were followed during 6 hours or more, which makes it possible to estimate dissociation rates in the order of $1 \cdot 10^{-6} \text{ s}^{-1}$. To the best of our knowledge, no other technology that monitors real-time kinetics is capable of quantifying such slow dissociation rates. For example, biophysical interaction analysis on purified proteins with surface plasmon resonance (SPR) is currently limited to approximately $1 \cdot 10^{-5} \text{ s}^{-1}$ [28].

Interaction Map analysis is a novel and powerful approach to evaluate and display time-resolved molecular interaction data. Even though Interaction Map is originally designed for the analysis of how one monovalent molecule interacts with a heterogeneous target, it can be applied also to the evaluation of a binding of a bivalent molecule like an antibody to a heterogeneous target. When violating the monovalent requirement of Interaction Map, however, the interpretation of the map as such has to be conducted with caution. The interaction between ^{131}I -panitumumab or ^{131}I -cetuximab and EGFR on A431 cells was characterized by one dominant binding process, indicating that the antibody interaction behaves like a monovalent interaction in these particular cases. This was supported by Interaction Map analysis of the ^{125}I -cetuximab-EGFR exposure time variation study that resulted in a binding affinity that was not dependent on exposure time. If more and more antibodies bind with its second epitope and stabilize its binding through this avidity effect, a gradual increasing affinity with prolongation of cetuximab exposure time would be expected.

Compared to EGFR binding on A431 cells, ^{131}I -panitumumab and ^{131}I -cetuximab binding to EGFR on HaCaT cells showed a more pronounced secondary interaction event (Figure 2, low intensity peak at the position: $\text{Log}_{10}(k_D) = -3$; $\text{Log}_{10}(k_a) = 5$). Based on the present data, it was not possible to elucidate the exact nature of heterogeneity in binding behavior of these

antibodies. With respect to heterogeneity of the binding process, however, both antibodies had a similar Interaction Map signature when binding to the same cell line. Therefore, our hypothesis is that the second low intensity peak for the antibody binding to EGFR on HaCaT cells represents something on the cell surface. For example, the presence of an independent epitope or a conformational change related to monomer/dimer status such as the presence of EGFR dimers without EGF stimulation [12].

In the eighties it became apparent that EGF activates its receptor by inducing dimerization [29-32] and the receptor mediated dimerization leads to a structural rearrangement that stabilizes the complex [21, 33]. Evidence that also unligated EGFR dimers or oligomers exist [34-38] and that affinity among EGFR is affected by mutations [39] adds to the complexity of the interaction processes. For the binding strength of EGF to EGFR on A431, low nM affinities have been reported for the dominating interaction and a 10-20-fold higher affinity for a secondary interaction [40, 41].

In the present study, Interaction Map analysis showed that the interaction between ^{131}I -EGF and EGFR was more heterogeneous than binding of the antibodies cetuximab and panitumumab. The Interaction Map of ^{131}I -EGF binding to EGFR on A431 resulted in a similar pattern as reported previously [8, 23], albeit with a slight difference in the relative proportions of the two different components. ^{131}I -EGF binding to EGFR on A431 had a more pronounced high affinity peak compared to previously reported interaction patterns [8]. The time exposure variation experiments with a relative high ^{125}I -EGF concentration, i.e., 30 nM which is more than 100 times higher than EGF levels in plasma [42], revealed that the relative contribution of the high affinity components increased as function of the exposure time. An increase in contribution from the high affinity component of the overall interaction has also been observed when A431 cells were treated with gefitinib and erlotinib, two inhibitors that are known to promote dimerization of EGFR family members [8, 23, 42]. These observations are in support of the ligand binding induced dimerization/oligomerization model as both increasing EGF concentrations and exposure time increase the change that ligated EGFR bind to each other on the cell surface in a conformation that has a higher affinity for EGF. These results clearly illustrate that the heterogeneity in the EGF-EGFR binding dynamics is not just dependent on the quantity of EGFR family members in their cellular environment but

that it also depends on EGF concentration and exposure time.

Literature values on kinetic rate constants and affinity constants of interacting proteins are often based on kinetic interaction analysis with more established biophysical techniques like SPR where one protein is covalently attached or captured on a sensor surface while the other protein is brought in contact with the sensor surface with typical exposure times of a few minutes [3, 5]. Studies in which EGF binding to the soluble extracellular ligand binding domain EGFR (sEGFR) were measured with SPR, resulted in affinities in the range of 100 to 500 nM for sEGFR monomers [19, 24, 32, 33, 43, 44] and a range of 5 to 60 nM for sEGFR dimers [24, 32, 44]. Reported association rates were in the range of $1.5 \cdot 10^5$ to $3.8 \cdot 10^5$ $M^{-1}s^{-1}$ for both EGF binding to sEGFR monomers and dimers. Dissociation rates for the EGF-sEGFR monomer were in the range of 0.041 to 0.066 s^{-1} and slower for EGF-sEGFR dimers ranging from 0.001 to 0.013 s^{-1} [24, 32, 44].

Comparing interaction rates derived from cell- and biophysical-based techniques, it becomes clear that interaction kinetics is not only cell line dependent but also method dependent. In the present study, a 3 minute exposure of ^{125}I -EGF to EGFR to A431 cells resulted in an association rate for the high affinity interaction above $1 \cdot 10^5$ $M^{-1}s^{-1}$ which is close to reported association rate for interactions with purified proteins. This association rate, however, gradually decreased to below $2 \cdot 10^4$ $M^{-1}s^{-1}$ if exposure times were prolonged to half an hour or longer. When comparing the EGF-EGFR dissociation rates, the difference is more pronounced with a 10-100 times slower dissociation rate obtained from cell-based measurements. Dissociation rates differing two orders of magnitude are also seen when comparing the present cetuximab-EGFR interaction with literature values for cetuximab binding to purified sEGFR [44]. This technique based difference in measured dissociation rates is not only limited to proteins binding to EGFR but has also been observed before, for example Nilvebrant *et al.* who investigated antibody fragment binding to CD44v6 [27]. The higher binding stability for interactions that take place on living cells has an interesting implication for using *in vitro* data to predict the pharmacokinetics and efficacy of drugs *in vivo*. Recently, in an article by Dahl and Akerud, the usage of drug-target residence time information was questioned because many successful drugs have ligand-target dissociation rates that are too fast to affect the duration of drug effectiveness [2]. This

conclusion was based on a comparison of *in vitro* ligand-target half-life values, as measured with biophysical techniques, and *in vivo* drug elimination, based on plasma half-life studies. For example, the Lapatinib-EGFR/HER2 interaction had a dissociation half-life estimated using biophysical techniques that was 5 times lower than the plasma half-life [1, 45]. The empirical experience from time-resolved cell based assays that dissociation rates are typically a factor of 10-100 slower than the rates obtained with assays that utilize purified proteins means that the drug-target residence time might be longer than the pharmacokinetic (PK) elimination time and thus might constitute the dominating factor that should be considered when optimizing the duration of drug effectiveness. Thus characterization and optimizing of drug-target residence time using a protein-protein interaction assay might still be valuable for drug development. At the same time, differences between cell lines denote the importance of validating the results in living systems [46, 47]. In particular, as it has been shown that ranking on dissociations rates, obtained from biophysical techniques, sometimes show remarkable differences when comparing ranking results obtained with a cell based assay [27].

The cellular environment provides a dynamic environment for interactions to take place which, in turn, can affect the kinetic properties. Three potential processes that affect interactions kinetics are; 1) apparent binding rates might be reduced because ligands require time to diffuse to and from the more obstructed receptors located between cells in a confluent cell layer meaning that the local ligand concentrations is not the same as the ligand concentration in solution, 2) the association rate might be slowed down because receptors are limited to a two dimensional motion thereby reducing the rate of meeting its ligand compared to interaction conditions in solution and 3) interactions might be stabilized through a binding induced change in the environment leading to a two-state process. The EGFR dimerization is an extreme example of such a two-state process and by using the Interaction Map model these two interaction states could be characterized individually [12]. Cellular processes like internalization might also play a role, especially as EGF internalization is receptor mediated [48]. However, in a previous study it was shown that although internalization occurs at room temperature, no degradation was observed and it was concluded that internalization was balanced by recycling as internalization did not significantly affect EGF-EGFR

binding kinetics [23]. There is currently insufficient evidence to pinpoint if any of these above mentioned processes alone or in combination cause the observed differences in interaction characteristics.

Patient differences in the response to anti-EGFR cancer treatment are intensively studied in current research. Increased levels of receptor ligands, co-expression of EGFR mutants, cross-talk with HER2 or other receptors, and exposure to various EGF levels are mechanisms that can alter EGFR signaling output and potentially alter the response to EGFR inhibitors [13, 49]. Unfortunately, current research methods are not able to reveal, display or analyze these drug development obstacles [13]. In this report, we showed in detail that the two therapeutic antibodies had clear kinetic differences between the two employed cell lines, which we believe can be related to variations in response to drug efficacy. In addition, the current study illustrated that EGF concentrations is a factor to take into account when studying drugs that compete for the EGF binding site as the stability of EGF ligation and thus EGFR occupancy is dependent on the EGF concentration.

CONCLUSION

In conclusion, this study explores the combination of methods (LigandTracer and Interaction Map) which provides interaction characteristics on living cells in high detail. The ability to measure interaction characteristics and interaction complexity on living cells is a requirement to advance the understanding of ligand-receptor interactions, especially when it involves heterogeneous interactions where the dynamics in heterogeneity is affected by the interaction itself as seen for EGF-EGFR binding. Also the binding of two therapeutic antibodies to cellular receptors on two cell lines revealed interesting differences. The affinity of panitumumab was higher than that of cetuximab but the same magnitude of difference in affinity was observed when comparing binding of the same antibodies to A431 and HaCaT cells. Moreover, the heterogeneity of antibody binding, as revealed by Interaction Map analysis, was primarily dependent on the cellular context.

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