

## Binding Kinetics on OptiSlides

### Characterization of polyclonal anti-rabbit IgG

#### Introduction

In the field of proteomics, one has to deal with a class of molecules with diverging and in several aspects still unpredictable properties. It is quite undesirable to have to modify proteins with labels before being able to characterize them. Obviously, under such circumstances label-free detection methods are strongly favoured.

Surface plasmon resonance spectroscopy is the most common label-free technique. However, a gold layer is needed on the solid support which has to be produced under highest standards. Current applications are in most cases restricted to a few regions of interest. Protein arrays on standard glass slides cannot be read out or even be characterized kinetically.

The quantification of the affinity of an antibody to its partners and the evaluation of its unspecific binding is an important issue. With the Imaging Ellipsometer EP<sup>3</sup> the interaction of an antibody to immobilized antigens on a microarray can be observed in a time resolved, label-free and highly parallel manner [1]. Today, up to 150 channels, measured at so called 'regions of interest' (ROI), can be analyzed simultaneously. Different from common SPR setups, the binding of a ligand to several receptors can be studied in one experiment and thus consistent biochip data is obtained. Using Nanofilm's proprietary OptiSlides, a very high sensitivity can be reached. Here we show the determination binding constants of polyclonal anti-rabbit IgG to immobilized rabbit IgG and study the specificity of the polyclonal antibody.

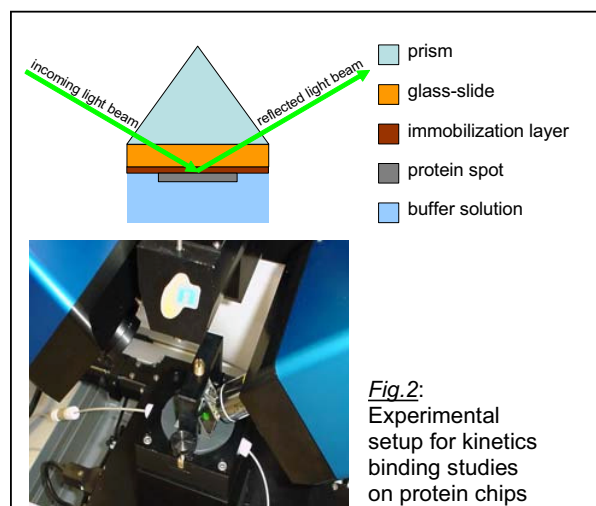
#### Sample

OptiSlides manufactured by UNAXIS (Balzers, Liechtenstein) were covered with a Dendrimer surface and spotted with rabbit IgG (first series) and five proteins

(second series of experiments) at Chimera Biotech (Dortmund, Germany; array layout in fig.1). Phosphate buffered saline (0.01 M phosphate buffer, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) with 0.005% Tween was used as running buffer.

#### Instrumentation

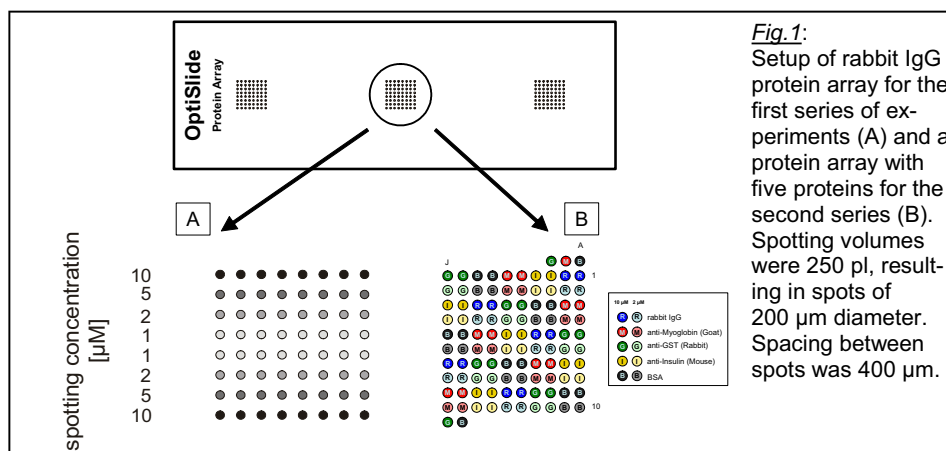
Standard Imaging Ellipsometer EP<sup>3</sup>-SW (532 nm), beam expander & 2x objective, field of view: 2.1 x 2.7 mm, Kinetics / SPR cell with 60° BK7 prism (n=1.5), peristaltic pump, EP<sup>3</sup> View Software V2.03 with Kinetics AddOn module



**Fig.2:**  
Experimental setup for kinetics binding studies on protein chips

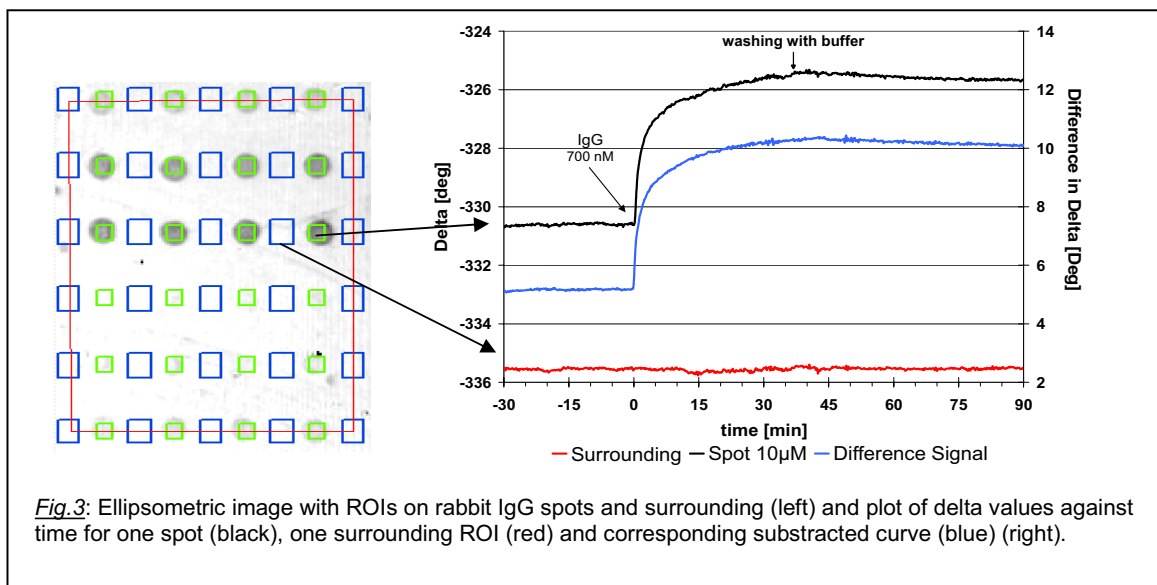
#### Task

In the first part the determination of kinetic constants for binding of polyclonal anti-rabbit IgG to immobilized Rabbit IgG is examined and in the second part the specific and unspecific binding of it to 5 immobilized antigens is evaluated.



**Fig.1:**  
Setup of rabbit IgG protein array for the first series of experiments (A) and a protein array with five proteins for the second series (B). Spotting volumes were 250 pl, resulting in spots of 200 µm diameter. Spacing between spots was 400 µm.

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## Steps of evaluation

1. Inserting the slide into the Kinetics / SPR cell.
2. Filling the cell with the running buffer. The buffer is pumped with a flow rate of 1 ml/min in a cyclic manner (volume: approx. 1.5 ml).
3. Visualization of the spots at 54° angle of incidence, adjustment of the optical components to get a good contrast.
4. Adjustment of the x/y position to spots of interest.
5. Definition of regions of interest (ROI) on the spots and around them as reference channels.
6. Measurement of the ellipsometric parameter delta on the spots and their surroundings. The difference between those provides information for calculating thickness or mass (fig. 3).
7. For the kinetics experiments defined concentrations (3 - 700 nM) of anti-rabbit IgG (goat) were pumped through the flow cell containing the IgG array (fig. 1A) in a cyclic manner (total volume here: approximately 1.5 ml) and binding was monitored by recording delta values in 10 seconds steps. For each concentration a new array was used. The data of 16 individual spots were chosen for evaluation.
8. For the analysis of specificity 50 nM anti-rabbit IgG (goat) solution was pumped through the flow cell containing the array shown in figure 1B. After washing with buffer to examine the offrate of the polyclonal anti-rabbit IgG the array was regenerated by washing with glycine buffer (pH 2.2) and PBS-Tween.

## Results

## Kinetics experiments

As shown in fig. 3, the binding of anti-rabbit IgG is significant and quick, indicating a high  $k_{on}$  value. On the other hand, dissociation is very slow (see slope during the washing step). As a consequence, though calculation of  $k_{off}$  is possible and has been shown, for more accurate calculation of kinetic constants the following approach has been applied:

1. The association was measured at various ligand concentrations.
2. The exponential rate constant  $\tau$  for each concentration was estimated with a fitting routine (implemented in the Kinetics AddOn software) following:

$$\Delta f(t) = \Delta f_{equ} \left( 1 - \exp\left(-\frac{t}{\tau}\right) \right) + a \cdot t$$

where  $a \cdot t$  is a correction term for biphasic reactions.

3.  $1/\tau$  is plotted against the analyte concentration  $c_{L,0}$ . The slope of the curve is the association rate constant  $k_{on}$ , the intercept with the y-axis is the dissociation rate constant  $k_{off}$ :

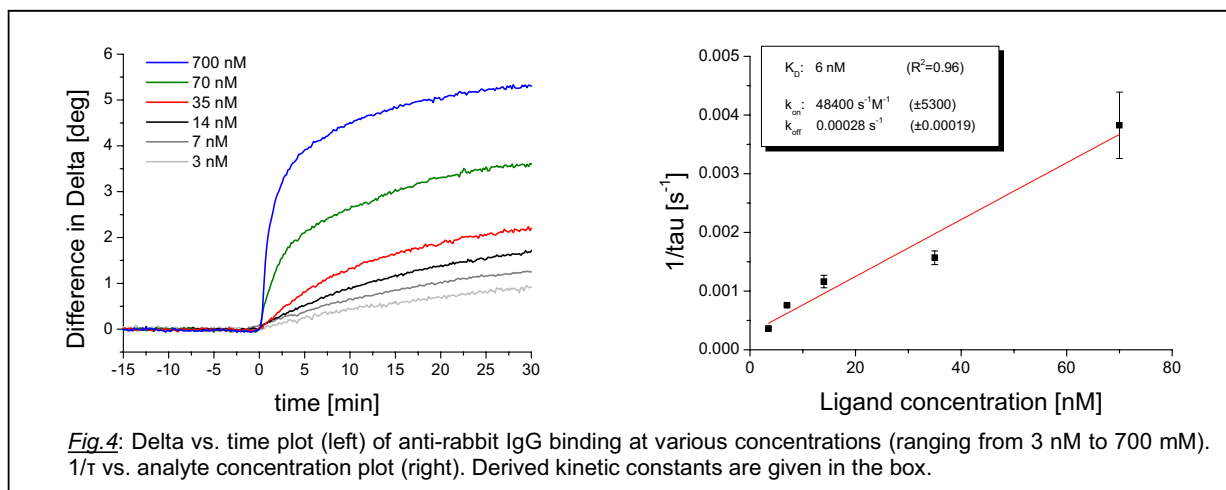
$$\frac{1}{\tau} = k_{on} c_{L,0} + k_{off}$$

4. The binding constant  $K_D$  is the quotient of  $k_{off}$  and  $k_{on}$ :

$$K_D = \frac{k_{off}}{k_{on}}$$

A series of binding curves, the corresponding plot of  $1/\tau$  and derived kinetic constants are shown in fig. 4.

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As mentioned above, kinetics can be followed for each single protein spot, independently from the amount of protein spotted. This has been shown by calculating kinetic constants for a series of 16 spots arranged as in fig. 1 and 3. A record of delta values for these spots bound by 7 nM IgG plotted against time is shown in fig. 5. One can see that even for the spots of lowest concentration a calculation of kinetic constants is possible (see also 0.5  $\mu\text{M}$  spots curves in fig. 5, right).

Optical modeling allows the determination of the refractive index of any material analysed. Here the thickness of individual spots for a three-dimensional visualization of the chip surface was determined.

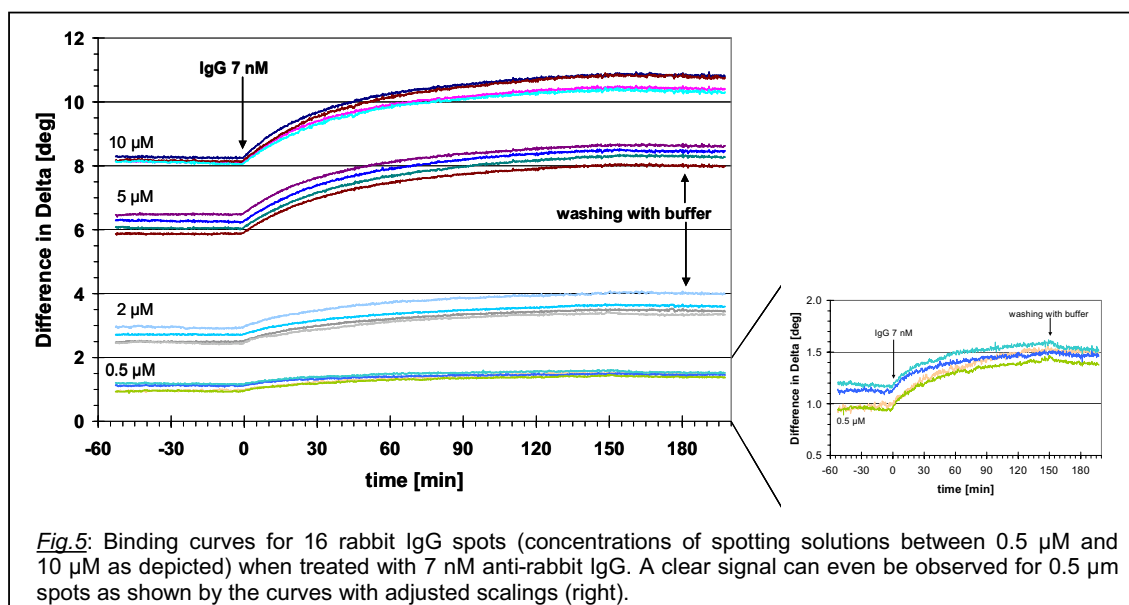
Fig. 6 shows a rabbit IgG array before and after binding of anti-rabbit IgG.

Specificity of the polyclonal antibody

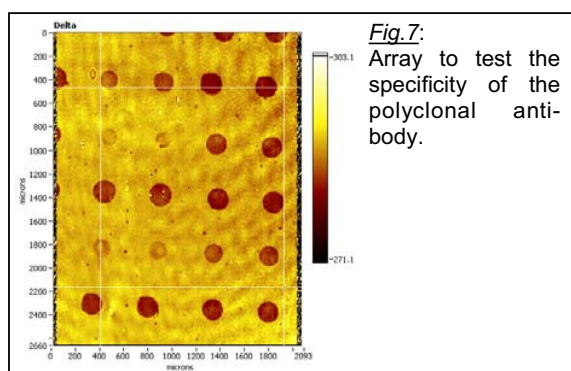
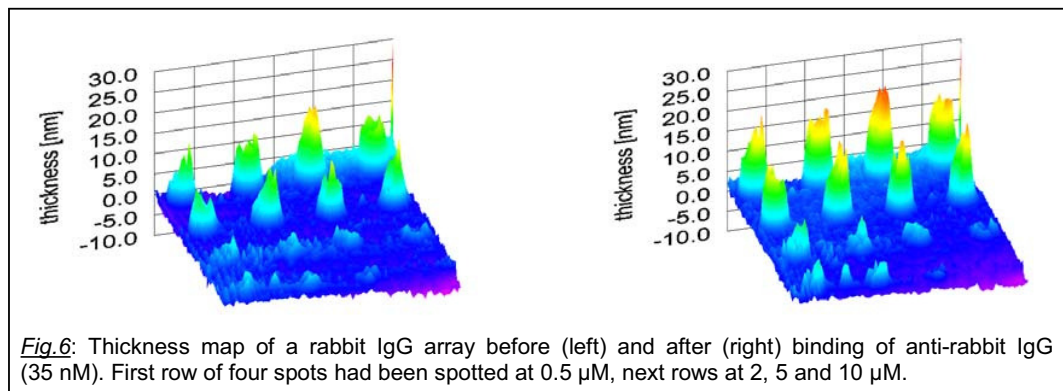
In a second series of experiments the specificity of the polyclonal anti-rabbit antibody has been examined. An array with five proteins, four antibodies and serum albumin in two concentrations, has been printed as on fig. 1.

With an EP<sup>3</sup> equipped with the 2x objective, an area of 2 x 3 mm can be examined, corresponding to 4 x 5 spots (fig. 7).

The anti-rabbit IgG bound specifically to rabbit antibodies. (fig. 8). No binding could be observed to anti-myoglobin IgG from goat, anti-insulin IgG from mouse or to bovine serum albumin.



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## Acknowledgement

We would like to thank Unaxis for their cooperation developing the OptiSlides and Chimera Biotec for supplying the samples.

## Conclusion

The Imaging Ellipsometer EP<sup>3</sup> is an attractive alternative for label-free kinetics analysis. The interaction of a soluble ligand with immobilized receptors on a protein array has been demonstrated. Using OptiSlides as a substrate overcomes limitations of SPR based systems in terms of linker chemistry. It has been shown that each individual protein spot can be evaluated achieving consistent kinetic constants. Different protein array samples have been used in this study, leading to consistent, reproducible data sets.

## Literature

- [1] Vaupel et al., in "Microarray Technology and Its Applications", Müller & Nicolau (Eds.), Springer, Berlin **2005**, ISBN 3-540-22931-0, 181-207
- [2] Angenendt et al., *Journal of Chromatography A*, **1009 (2003)** 97-104
- [3] Yasui, in "Real-Time Analysis of Biomolecular Interactions", Nagata & Handa (Eds.) Springer, Tokyo **2000**, ISBN 4-431-70289-X

