# Chapter 13

# Reflectometric Interference Spectroscopy

**Guenther Proll, Goran Markovic, Peter Fechner, Florian Proell, and Guenter Gauglitz**

## Abstract

Reflectometry is classified in comparison to the commercialized refractometric surface plasmon resonance. The advantages of direct optical detection depend on a sophisticated surface chemistry resulting negligible nonspecific binding and high loading with recognition sites at the biopolymer sensitive layer of the transducer. Elaborate details on instrumental realization and surface chemistry are discussed for optimum application of reflectometric interference spectroscopy (RIfS). A standard protocol for a binding inhibition assay is given. It overcomes principal problems of any direct optical detection technique.

**Key words** Label-free optical biosensor, Reflectometric interference spectroscopy (RIfS)

## 1 Introduction

The methods available for direct monitoring of biomolecular interaction can be divided into methods measuring changes in the refractive index of the interaction layer, and methods measuring changes in reflectometry at the layer [1]. Regarding the first method, BiaCore [2] has opened the market by introducing surface plasmon resonance (SPR) [3] as a very promising tool in biomolecular interaction analysis (BIA). In contrast to refractometry, reflectometry concentrates on the measurement of changes in optical thickness. Reflectometry has been introduced many decades ago as ellipsometry using polarized light. Interference at the interfaces of the layer causes a change in the relative amount of amplitude of the two polarized radiation beams and in phase. This interferometric method has been applied to a very simple analytical method, called reflectometric interference spectroscopy (RIfS) to be used as an example of a very robust, simple optical detection principle in chemical and biochemical sensing [4]. This label-free optical detection method for surface interactions is based on white light interference at transparent thin layers. At each interface of thin layers of

Avraham Rasooly and Ben Prickril (eds.), *Biosensors and Biodetection: Methods and Protocols Volume 1: Optical-Based Detectors*, Methods in Molecular Biology, vol. 1571, DOI 10.1007/978-1-4939-6848-0_13,

different materials with negligible absorption, radiation is partially reflected and transmitted. If the optical path length through these layers is less than the coherence wavelength, the different partial beams interfere, and form an interference pattern depending on the wavelength, the optical thickness which is given by the product of the refractive index of the layer and its physical thickness, the incident angle, and the refractive index of the surrounding medium [5, 6]. In case of perpendicular incidence, a non-absorbing layer, and low reflectances, the reflectance R is given by:

$$R = R_1 + R_2 + 2\sqrt{R_1 R_2}\cos\left(4\pi\, nd/\lambda\right) \tag{1}$$

where $R_1$ and $R_2$ denote the Fresnel reflectance at the two interfaces, $d$ is the physical thickness of the film, $n$ its refractive index, and $\lambda$ the wavelength of incident light (*see* **Note 1**). A typical interference pattern showing the modulation of reflectance with $\cos(1/\lambda)$ is given in Fig. 1.

The optical thickness $n{\cdot}d$ can be determined from the position of an extremum with a given order value $m$ by

$$n \times d = \frac{m\lambda}{2}. \tag{2}$$

RIfS uses the change in the optical properties in or at the top layer of a given layer system as detection principle (Fig. 1). The binding of an analyte molecule or particle to the sensor surface causes a shift of the interference pattern in the wavelength domain. To evaluate the binding signal, the locus of an extremum is tracked over time; thus, the change of the interference spectrum results in a time-resolved binding curve representing the binding of the analyte molecule to the sensor surface.

A major advantage of RIfS is its resistance to changes in temperature [7]. Refractometric methods such as SPR and ellipsometry, on the other hand, are very sensitive to temperature variations due to the high impact of temperature on the refractive index. Thus, temperature changes during a measurement cause negative effects with these methods, and quick changes of temperature between measurements are technically challenging. Since the

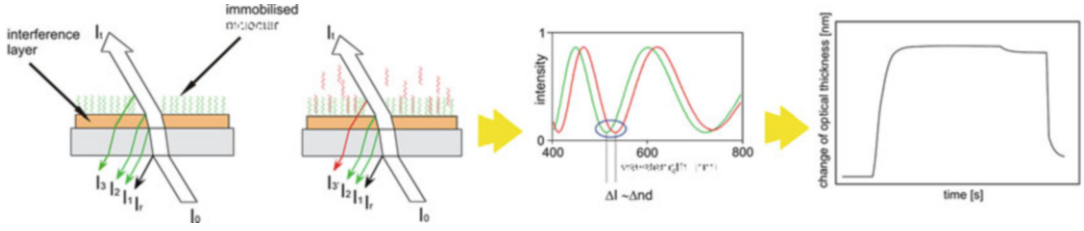


**Fig. 1** Scheme of the RIfS detection principle. The *left part* shows the superimposition of the reflected light beams and the change in optical thickness during a binding event on the sensor surface. The *right part* shows the corresponding change of the characteristic interference spectrum and the resulting binding curve

refractive index $n$ is dependent on the density given from the Clausius Mossotti equation, and the density is dependent on the thermal expansion, the refractive index decreases with increasing temperature. Due to the thermal expansion of the biopolymer-layer, the physical thickness $d$ increases with increasing temperature. These two contrary temperature-dependent effects result in a rather low influence of temperature on the optical thickness, the product of the refractive index and the physical thickness.

## 2 Materials

Common chemicals of analytical grade are purchased from Sigma or Merck. Milli-Q water is deionized water with a conductivity of 18.2 MΩ $cm^{-1}$.

### 2.1 Transducer

The RIfS standard transducer consists of a glass substrate (D 263 glass, Schott AG, Germany) (~1 mm thick) coated with a 10 nm layer of a material with high refractive index (usually $Ta_2O_5$ or $Nb_2O_5$), and a top layer of $SiO_2$ (330 nm). As a reference use a transducer without $SiO_2$ layer. Due to the technical specifications of the components used for setting up a RIfS measurement system and the investigated sample types (e.g., liquids or gases), other transducers might be more beneficial. An ideal RIfS transducer is designed to create the two reflected light beams of interest at similar intensities. Therefore, it is recommended to use ray tracing programs (e.g., FilmWizard by SCI Scientific Computing International or WVASE by J. A. Woollam) to simulate the expected reflectivities for the visible wavelength range for a given RIfS setup, taking into account the expected optical properties of the investigated sample types.

### 2.2 Surface Chemistry

1. GOPTS (3-glycidyloxypropyltrimethoxysilane) purum: Toxic. Store at room temperature, keep under Argon, sensitive to humidity (Fluka).
2. AMD (aminodextran): MW 100 kD, level of amination 50%. Store at 4 °C under dry conditions (Innovent e.V. Technologieentwicklung Jena, Germany).
3. Dicarboxypoly- and diaminopoly(ethylene glycol) (PEG): MW 2000 Da. Store at −20 °C under dry conditions (Rapp Polymere, Tuebingen, Germany).
4. NHS (N-hydroxysuccinimide) purum: Store under room temperature (Fluka).
5. DIC ($N,N'$-diisopropylcarbodiimide) purum: Toxic. Store at room temperature, keep under argon, sensitive to humidity (Fluka).

6. EMCS (6-maleimidohexanoic acid *N*-succinimidyl ester) purum: Store at −20 °C under dry conditions (Sigma).
7. TBTU (2-(1H-benzotriazol- 1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate).
8. HOBT (1-hydroxybenzotriazole) Hydrate purum: Sore at −20 °C at dry conditions (Fluka).
9. DIPEA (*N*,*N*-diisopropylethylamine): Highly inflammable. Store at room temperature (Sigma).

### 2.3 Glass Substrates

1. BK 7 glass, $n = 151$, Schott, Mainz.
2. WG 345 glass, $n = 1699$, Schott, Mainz.
3. Interference transducer: D 263, 10 nm $Ta_2O_5$, 500 nm $SiO_2$, Schott, Mainz.
4. Goethe glass: multi-layer system: 1 m D 23, 45 nm, $Ta_2O_5$, 20 nm $SiO_2$, Schott, Mainz (Cut in small squares (10 × 10 mm)).

### 2.4 Parallel Setup

1. White light source 100 W/12 V, Osram, Munich.
2. Lenses, mirror, and positioning optics, Spindler&Hoyer, Göttingen
3. Polymer light guides (PMAA, $n = 1490$, coupling element 1 × 2 (50:50), 1 mm, fiber diameter with SMA 905 fiber connectors, Microparts, Dortmund.
4. Optical 4-×-1 multiplexer DiCon VX 500-C, Laser Components, Olching.
5. MMS diode row spectrometer with Liliput-PC, ZEISS, Jena.
6. 19″ industrial standard housing by RS Components, Walldorf-Mörfelden.
7. Commonly Microsoft Windows XP, Vista, Windows 7, Windows 8, Windows 8.1, Windows 10 operating systems.

### 2.5 Single Setup

1. Modified simultaneous spectrometer SPECKOL 1100, Zeiss, Jena
2. Commonly Microsoft Windows XP, Vista, Windows 7, Windows 8, Windows 8.1, Windows 10 operating systems.

### 2.6 Advanced Single Setup (See Fig. 4)

1. Light source providing light of a single color (White light source 100 W/12 V, Osram, Munich with Bandpassfilter 568 nm).
2. Phodiode based detection unit (Lightwave-Multimeter 2832-C with Si-photodiodes of the series 818 from Newport).

### 2.7 Liquid Handling

1. Ten position valve VICI, Valco Europa, Schenkon, Switzerland.

2. HPCL 3-way valve VICI, Valco Europa, Schenkon, Switzerland.
3. Six position valve, Bischoff, Leonberg.
4. Peristaltic pump Reglo-Digital MS2/8-160 ISM 832, Ismatec, Wertheim.
5. Peristaltic pump MS Fixo, Ismatec, Wertheim.
6. High grade steel capillaries, screws and fittings from Rheodyne, USA.

### 2.8 Software

1. Measure$^{CR}$ for capturing spectras and controlling the systems. Depending on the selected spectrometer, any other software control can also be used. As most commercial available spectrometers are supplied with ready to use software or plug-ins for the widely used Lab-View solution (National Instruments Corporation), it is recommended to use one of these solutions.
2. IFZ$^{CR}$ for evaluating the interferograms. Any other software solution capable for performing the same simple mathematical calculations like Matlab (The MathWorks, Inc.) can be used.
3. MS-Excel (Microsoft) and Origin (MicroCal Inc.) or any equivalent statistical software packages (open source software is available) can be used for further processing of measured data.
4. If an advanced single color setup for reflectometry measurements is used, no spectras have to be analyzed. Therefore, software only needs to control hardware components. All evaluation work can be done with software packages like Matlab or Origin.

## 3 Methods

### 3.1 Setup for RIfS

In the standard laboratory setup for RIfS (Fig. 2), the white light is guided via an optical fiber (1 mm PMMA fiber) to the back of the transducer mounted in a microfluidic flow cell, which in turn is attached to a liquid handling system. The reflected light is gathered in the same waveguide.

The fiber optics used is bifurcated (50:50 ratio), with one tail leading to the light source and the other to the UV–Vis spectrometer. Possible light sources are halogen lamps (e.g., 10 W halogen lamp with fiber in-coupling optics consisting of front surface spherical mirror, collimating lens, and an infrared absorption filter) or LEDs. For the spectral detection of the reflected light, diode array spectrometers are used normally. A gap (approx. 100 μm) between transducer chip and the fiber output is filled with glycerol (80%) for refractive index matching. Samples are handled by a flow system (Fig. 3) (e.g., two peristaltic pumps, inject-load valve, and 6-way valve).

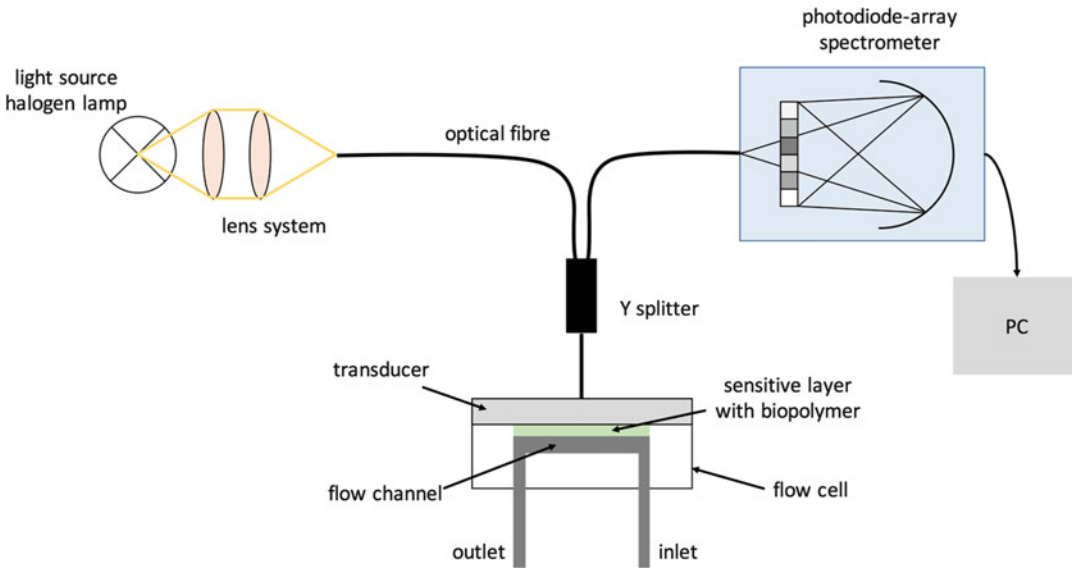


**Fig. 2** Classical RIfS setup

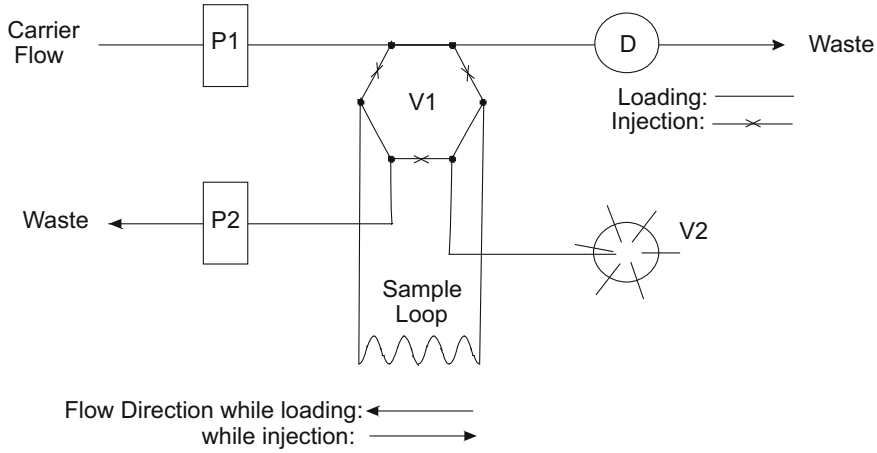


**Fig. 3** Schematic drawing of the FIA system for RIfS

Moreover, this flow system can be equipped with an autosampler. The various samples are injected by the autosampler into a sample loop. From there, the sample is driven in continuous flow, passing the prepared transducer.

The raw spectra are corrected for the dark current of the spectrometer by subtraction (if necessary), and normalized to the reflectance spectrum from a glass chip without the $SiO_2$- and biolayer. The position of an interference extremum at approx. 550 nm is determined by a parabolic fit to one halfwave of the interference spectrum. Optical thickness is calculated according to Eq. 2 (*see* **Note 2**).

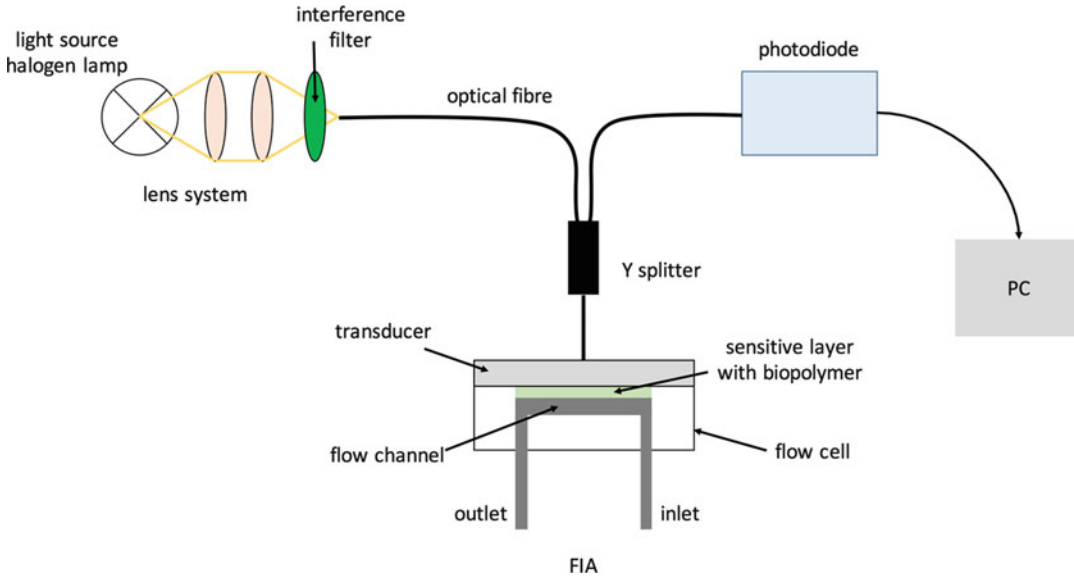


**Fig. 4** Advanced setup for single-color RIfS detection according to [15]

The costs of the setup can be reduced by sequentially illuminating the transducer with different suitable wavelengths coming from LEDs or a white light source with appropriate filters, detecting the intensity without spectral resolution using a photodiode, and reconstructing part of the interference pattern by fitting a parabola through the interpolation points. The advanced development is a single-color (wavelength) RIfS approach, which uses a color filtered light source (Fig. 4) or even a LED, monitoring the change in intensity looking at a distinct wavelength rather than the shift of an extremum (*see* Fig. 1). This allows a very simple instrumentation setup and the use of a second wavelength as reference.

This detection principle can be used to realize a parallel screening system which allows optical online detection of specific biomolecular interaction in 96- or 384-well microplate formats (*see* Fig. 5) Reflectometric interference spectroscopy (RIfS):parallel set-up. Therefore, the whole area of the plate bottom consisting of a RIfS transducer is illuminated by a halogen light source combined with a filter wheel, which allows the subsequent passage of monochromatic light of seven different wavelengths. This is not only to reduce the costs, but is necessary because a CCD camera is used as detector which is able to detect the intensity of the whole area of interest in a single shot.

Singe-color RIfS also has an advantage in a parallelized setup. A commercial imaging device was developed by Biametrics GmbH that can measure up to 20,000 events per square centimeter on the transducer under flow-through conditions while obtaining information on thermodynamics and kinetics in parallel.

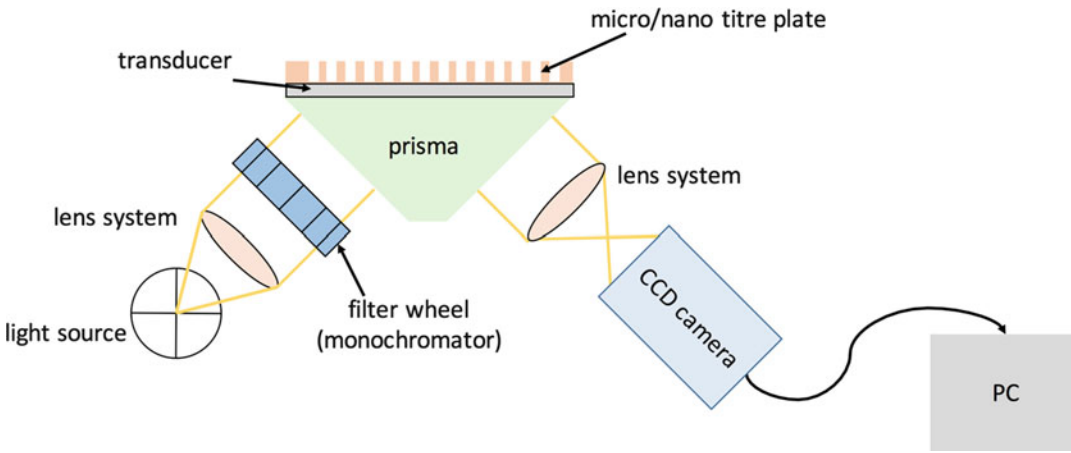


**Fig. 5** Parallel RIfS setup

### 3.2 Surface Chemistry

All surface chemistry steps are applied to the coated side of the RIfS transducer (*see* **Note 3**) if not stated otherwise.

#### 3.2.1 Silanization

(a) Transducer chips are cleaned with freshly prepared Piranha solution (mixture of 30% hydrogen peroxide and concentrated sulfuric acid at a ratio of 2:3; caution: hot and aggressive!) for 30 min in an ultrasonic bath to clean the chip and to generate silanol groups on the transducer surface. As an alternative, this activation step can be carried out using oxygen plasma.

(b) After rinsing with double distilled water and drying in a nitrogen stream, the surface is immediately activated by GOPTS at a surface concentration of approx. 10 µl/cm$^2$ for 1 h in dryness (by assembling two slides face-to-face placed in a tray with ground joint) followed by cleaning with dry acetone and drying in a nitrogen stream (*see* **Notes 4** and **5**).

#### 3.2.2 Biopolymer

When investigating interactions of biomolecules on surfaces, nonspecific binding to the sensor must be minimized. Therefore, the glass slide is coated with a shielding layer which prevents nonspecific binding and additionally provides a large number of functional groups for the immobilization of the binding partner. The standard shielding chemicals used are dextrans which form an 3D-hydrogel loaded with a large number of binding sites, and polyethylene glycols (PEG) forming a two-dimensional brush-like monolayer with less binding sites but with a more defined surface. Amino and carboxy groups are normally functional groups for the immobilization process since the well-established peptide chemistry methods can be applied. Other shielding chemicals can be used depending on the application or the surface chemistry needed.

(a) Aminodextran.
The coupling of aminodextran as an aqueous solution (1:3) (approx. 15 μL/cm$^2$) to the silanized surface is carried out by incubating over night (min 16 h) in a water-saturated atmosphere (*see* **Note 6**). After thoroughly rinsing with double distilled water and drying, the prepared chips are stable for several months.

(b) Polyethylenglycol.
The coupling of diamino- or dicarboxypoly(ethylene glycol) (PEG) as a 1 mM solution in dichloromethane (DCM) (approx. 10 μL/cm$^2$) to the silanized surface is achieved by incubating overnight at 70 °C, followed by thorough rinsing with Milli-Q water and drying in a nitrogen stream.

(c) Change of functional groups.
For some applications (e.g., immobilization of DNA oligomers with an amino-linker) the follow-up functionalization via peptide chemistry works better with diaminopoly(ethylene glycol) treated with 5 M glutaric anhydride (GA) in *N*,*N*-dimethylformamide (DMF) (10 μL/cm$^2$) for 6 h to generate carboxylic groups on the surface. The same protocol can be used to modify AMD-surfaces with carboxylic groups.

(d) One step chemistry.
Depending on the expected properties of the biopolymer layer, complex silanes offering chemically beneficial side chains like 11-aminoundecyltrimethoxysilane can be used in a one step procedure combining silanization with biopolymer coating.

### *3.2.3 Immobilization of Amino Terminated Ligands*

(a) The covalent coupling of amino-terminated molecules/ligands to biopolymers with carboxylic groups is done by standard peptide chemistry via activated esters (*see* **Note 7**). Therefore, the previously modified transducers are activated with a solution of NHS and DIC (1 M NHS, 1.2 M DIC in DMF, 10 μL/cm$^2$) for 2–4 h as sandwich pairs in a DMF saturated atmosphere (*see* **Note 5**). The transducers are rinsed first with DMF and then with dry Aceton, and dried in a nitrogen stream (*see* **Notes 4** and **5**).

(b) To achieve a high density of covalently bound molecules, it is necessary to use an excess of reagents. Therefore, the ligand is applied as a DMF solution (10 μL/cm$^2$) (if your ligand is insoluble in DMF, use another aprotic solvent of appropriate polarity) at a concentration of 1–3 μM to the activated surface for 4 h as sandwich pairs in a DMF saturated atmosphere (*see* **Note 6**). Clean the transducers by rinsing them with DMF and Milli-Q water and drying them in a nitrogen stream (*see* **Note 4**).

*3.2.4 Immobilization of Carboxy Terminated Ligands*

(a) For activation of the carboxy-terminated molecules, the ligand is dissolved in DMF at a concentration of 1–3 μM together with 1.5 M DIC. After quick mixing, this solution is immediately applied to a transducer modified with a biopolymer providing amino groups at a concentration of 10 μL/$cm^2$. Place a second transducer on top to form a sandwich pair (*see* **Note 6**).

(b) The reaction is finished after 4 h in a DMF saturated atmosphere (*see* **Note 6**). Clean the transducers by rinsing them with DMF and Milli-Q water and drying them in a nitrogen stream (*see* **Note 4**).

*3.2.5 Immobilization of Thiol Terminated Ligands*

(a) Dissolve 1 mg EMCS per 10 μL DMF and apply this solution to a transducer modified with a biopolymer offering amino groups at a surface concentration of 10 μL/$cm^2$ for 6–12 h in a DMF saturated atmosphere (*see* **Note 6**). After rinsing with DMF (*see* **Note 4**), dissolve the ligand at a concentration of 1–3 μM in DMF, and apply this solution to the transducers at a surface concentration of 10 μL/$cm^2$ for 6–12 h as sandwich pairs in a DMF saturated atmosphere (*see* **Note 6**).

(b) Clean the transducers by rinsing them with DMF and Milli-Q water and drying them in a nitrogen stream (*see* **Note 4**).

*3.2.6 Immobilization of Biotin*

(a) Biotin is immobilized by TBTU activation: D-biotin (1 mg, 4 mmol), TBTU (1.4 mg, 4.4 mmol), and DIPEA (4 mL, 23.3 mmol) are mixed with DMF (50 mL) until the active ester is formed and the reaction mixture appears homogeneous. This solution is dripped on to a transducer at a surface concentration of 10 μL $cm^{-2}$ pretreated with a biopolymer providing amino groups. Two of such transducers are put together to form a sandwich.

(b) After a reaction time of 4 h in a saturated DMF atmosphere (*see* **Note 6**), the sandwich is separated and both transducers are rinsed with water and dried in a nitrogen stream.

### *3.3 Measurement Protocol for Standard Binding Inhibition Assay for the Determination of the Affinity of an Antigen–Antibody Interaction*

Binding inhibition assay to determine the affinity constant of antigen-antibody interaction: The transducer is modified according to protocols given before either with a carboxy or an amino terminated antigen. Then fixed amounts of antibody are pre-incubated with fixed volumes of differently concentrated antigens (*see* **Note 8**) to reach binding equilibrium before injection of the mixture with the FIA system (*see* **Notes 9–12**) on the transducer.

Antigens with affinity to the antibody in the pre-incubated solution inhibit the binding of the antibody to the surface immobilized antigen. In the case of diffusion (mass transport) limited binding, the diffusion rate obeys first Fick's law. This results in a

constant binding rate of the antibody to the surface and a linear binding curve. The slope of the binding curve is determined by the concentration of free antibody binding sites in solution. The slope of the binding curves decreases with increasing concentration of antigen in solution and reaches zero for high antigen concentrations. Ovalbumin (final concentration of 200 µg/ml) should be added to all antibody solutions to avoid loss of antibody by nonspecific binding in the fluidic system. The model function describing the concentration of antibodies with free binding sites is fitted to the titration curve using a Marquart–Levenberg nonlinear least-square algorithm (software ORIGIN from Microcal, Northampton/USA) .

### 3.4 Data Evaluation

#### 3.4.1 Determination of the Concentration of Active Antibody

Measuring the concentration of active antibody in a sample working at mass-transport limited conditions is essential. Accordingly, the rate of diffusion to the surface is much slower than the binding reaction to the surface. The reaction kinetics can therefore be neglected. Mass-transport limited conditions can be achieved by a high binding capacity of the surface and a low antibody concentration in solution. Under mass-transport limited conditions according to first Fick's law, the resulting binding curve is linear, and its slope is proportional to the concentration of functional antibody. If all samples contain the same amount of protein, determined by UV spectroscopy or Bradford assay, it is possible to calculate the active antibody concentration in a.u. by the different slopes of the samples, where the sample with the highest slope is assigned the a.u. 1 per µg used protein.

#### 3.4.2 Determination of Affinity and Kinetic Constants

To determine the affinity and the kinetics of an antibody (*see* **Note 13**), mass-transport to the surface must be much faster than the rate of binding to the surface. If this is the case, the mass-transport can be neglected. This can be achieved by a high concentration of bulk antibody and a low surface coverage of hapten derivative.

The time dependence of the surface coverage can then be described by a pseudo first-order binding reaction as following:

$$\frac{\mathrm{d}\Gamma(t)}{\mathrm{d}t} = k_\mathrm{a} \cdot c_\mathrm{Ab} \cdot (\Gamma_\mathrm{max} - \Gamma(t)) - k_\mathrm{d}\Gamma(t) \qquad (3)$$

with $c_\mathrm{Ab}$: antibody concentration
$\Gamma(t)$: surface coverage at time $t$.
$\Gamma_\mathrm{max}$: maximal surface coverage.
$k_\mathrm{a}$: association rate constant.
$k_\mathrm{d}$: dissociation rate constant.

Solving this differential equation, the surface coverage which is equal to the resulting signal curve is given by:

$$\Gamma(t) = \Gamma_{\mathrm{Eq}}\left(1 - \mathrm{e}^{-k_{\mathrm{obs}}t}\right) \tag{4}$$

with.

$$\Gamma_{\mathrm{Eq}} = \Gamma_{\mathrm{max}} \frac{K_{\mathrm{aff}} \times c_{\mathrm{Ab}}}{1 + K_{\mathrm{aff}} \times c_{\mathrm{Ab}}}, \tag{5}$$

the equilibrium coverage and.

$$k_{\mathrm{obs}} = k_{\mathrm{a}} \times c_{\mathrm{Ab}} + k_{\mathrm{d}}, \tag{6}$$

the observed rate constant.

The affinity constant $K_{\mathrm{aff}}$ is given by the fraction of the association rate constant, $k_{\mathrm{a}}$, and the dissociation rate constant, $k_{\mathrm{d}}$.

To obtain accurate values for $\Gamma_{\mathrm{Eq}}$ and $k_{\mathrm{obs}}$, it is necessary that only the kinetics-controlled part of the signal curve is approximated, and that the surface and bulk are nearly in equilibrium at the end of the measurement. Now it is possible to determine $k_{\mathrm{a}}$ as the slope of the straight line of the plot with $k_{\mathrm{obs}}$ as ordinate and active antibody concentration as abscissa. In order to determine $K_{\mathrm{aff}}$ Eq. 5 can be rewritten as

$$\frac{1}{\Gamma_{\mathrm{Eq}}} = \frac{1}{\Gamma_{\mathrm{max}}} + \frac{1}{K_{\mathrm{aff}} \times \Gamma_{\mathrm{max}}} \times \frac{1}{c_{\mathrm{Ab}}}. \tag{7}$$

With this formula, it is possible to obtain the value for $\Gamma_{\mathrm{max}}$ with a linear fit of the $\frac{\frac{1}{\Gamma_{\mathrm{Eq}}}}{\frac{1}{c_{\mathrm{Ab}}}}$ plot. Inserting this value in Eq. 3, a nonlinear least square fit results in a value for $K_{\mathrm{aff}}$. This method gives nearly the same values as a Scatchard plot $\left(\frac{\frac{\Gamma_{\mathrm{Eq}}}{c_{\mathrm{Ab}}}}{\Gamma_{\mathrm{Eq}}}\right)$.

## 4 Notes

1. Very thick biolayers (more than approx. 100 nm) lead to measurements out of the linear correlation between change of optical thickness and shift of the interference spectrum.
2. Correct side of the RIfS transducers: mark the un-coated side with a diamond pen to avoid mistakes during the surface chemistry steps. The coated side of the transducers appears colored because of the light interference.
3. In the case a transducer shows a grey shadow after rinsing and drying repeat this cleaning procedure. If this does not work, start the surface chemistry from the beginning (otherwise the modification will not be homogeneous and will show high nonspecific binding).
4. All surface chemistry protocols which produce highly reactive groups are sensitive to humidity (deactivation). This can be

avoided by immediately applying the next step and working under dry ambient conditions.

5. Use a tray with ground joint. Place the transducer as sandwich-pairs (by assembling two slides face-to-face) in the tray on a solid support and add the same solvent which is used in the reaction for a solvent-saturated atmosphere.
6. In the case of limited ligands it is possible to use commercially available piezo-based microdosing devices for printing a ligand solution in Milli-Q water.
7. Usually phospahete buffered saline (PBS, 150 mmol NaCl and 10 mmol Di-potassiumhydrogenphosphate in Milli-Q water at pH 7.4) is used for antigen-antibody interaction analysis. In general all buffers can be used for RIfS measurements. Only restriction: do not use buffers with pH >8.5 because of destruction of the surface modification.
8. Testing of the modified transducer for nonspecific binding: use ovalbumine (OVA) or bovine serum albumin (BSA) in excess (e.g., 1 mg/mL) directly before the measurement.
9. Air disturbs the measurements: Use degassed buffers and avoid negative pressure (sucking) through the flow cell. In addition, it is helpful to keep the buffer under a slight positive argon pressure.
10. If possible use the same buffers during a complete measurement to avoid artifacts because of changes in the refractive index.
11. After the interaction process, the transducer surface can be regenerated. To remove antibodies from their antigens, a solution of 0.5% SDS (sodium dodecyl sulfate) at pH 1.9 is applied via the FIA system. In the case of hybridization experiments, a solution of either 0.25% SDS at pH 2.5 or a solution containing 6 M guanidinium hydrochloride and 6 M urea at pH 2 can be used.
12. RIfS is not restricted for biosensing; this technique can be used also for chemical sensing. Instead of biopolymers one can modify the transducer with for example nano-porous polymer layers, rubber like polymers or molecular imprinted polymers (MIPs). The determination of kinetics is also possible.
13. Nowadays, even more advanced devices based on the one-color approach are commercial available. These devices use the revised reflectometry read out called "1-lambda RIDe" (1-lambda Reflectometric Interference Detection). Currently, for example an imaging based version for the label-free readout of microarrays in the standard microarray format (b-screen®) is available from Biametrics GmbH. This technology enables the label-free detection of any microarray experiment like protein

or peptide microarrays, resulting in multiplexed kinetic experiments of up to 20,000 interactions in a single measurement.

14. Label-free detection like RIfS can be used for a great variety of applications ranging from standard kinetic analysis [8] to fragment-based screening [9]. New fields of application include the detection of small molecules by molecular imprinted molecules [10] or diagnostic applications [11–14].

## References


1. Gauglitz G (2010) Direct optical detection in bioanalysis: an update. Anal Bioanal Chem 398 (6):2363–2372
2. www.biacore.com
3. Homola J, Yee SS, Gauglitz G (1999) Surface plasmon resonance sensors: review. Sens Actuators B 54:3–15
4. Schmitt HM, Brecht A, Piehler J, Gauglitz G (1997) An integrated system for optical biomolecular interaction analysis. Biosens Bioelectron 12:219–233
5. Brecht A, Gauglitz G, Nahm W (1992) Interferometric measurements used in chemical and biochemical sensors. Analysis 20:135–140
6. Brecht A, Gauglitz G, Kraus G, Nahm W (1993) Chemical and biochemical sensors based on interferometry at thin layers. Sens Actuators 11B:21–27
7. Proell F, Moehrle B, Kumpf M, Gauglitz G (2005) Label-free characterisation of oligonucleotide hybridisation using reflectometric interference spectroscopy. Anal Bioanal Chem 382(2):1889–1894
8. Fechner P, Pröll F, Albrecht C, Gauglitz G (2011) Kinetic analysis of the estrogen receptor alpha using RIfS. Anal Bioanal Chem 400 (3):729–735
9. Pröll F, Fechner P, Proll G (2009) Direct optical detection in fragment-based screening. Anal Bioanal Chem 393(6–7):1557–1562
10. Kolarov F, Niedergall K, Bach M, Tovar GEM, Gauglitz G (2012) Optical sensors with molecularly imprinted nanospheres: a promising approach for robust and label free detection of small molecules. Anal Bioanal Chem 402(10):3245–3252
11. Ewald M, Le Blanc AF, Gauglitz G, Proll G (2013) A robust sensor platform for label-free detection of anti-Salmonella antibodies using undiluted animal sera. Anal Bioanal Chem 405(20):6461–6469. doi:10.1007/s00216-013-7040-9
12. Krieg AK, Gauglitz G (2014) An optical sensor for the detection of human pancreatic lipase. Sens Actuators B 203:663–669. doi:10.1016/j.snb.2014.07.036
13. Ewald M, Fechner P, Gauglitz G (2015) A multi-analyte biosensor for the simultaneous label-free detection of pathogens and biomarkers in point-of-need animal testing. Anal Bioanal Chem 407(14):4005–4013. doi:10.1007/s00216-015-8562-0
14. Rau S, Hilbig U, Gauglitz G (2014) Label-free optical biosensor for detection and quantification of the non-steroidal anti-inflammatory drug diclofenac in milk without any sample pretreatment. Anal Bioanal Chem 406 (14):3377–3386. doi:10.1007/s00216-014-7755-2
15. Frank R, Möhrle B, Fröhlich D, Gauglitz G (2005) A lable-free detection method of biochemical interactions with low-cost plastic and other transperent transducers. Proc SPIE Int Soc Opt Eng 5826:551–560