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# Analysis of cell surface antigens by Surface Plasmon Resonance imaging

Ivan Stojanović [a,*], Richard B.M. Schasfoort [a,b], Leon W.M.M. Terstappen [a]

[a] Medical Cell Biophysics Group, MIRA institute, Faculty of Science and Technology, University of Twente, PO Box 217, 7500AE Enschede, The Netherlands
[b] IBIS Technologies B.V., Pantheon 5, 7521PR Enschede, The Netherlands

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ABSTRACT

Surface Plasmon Resonance (SPR) is most commonly used to measure bio-molecular interactions. SPR is used significantly less frequent for measuring whole cell interactions. Here we introduce a method to measure whole cells label free using the specific binding of cell surface antigens expressed on the surface of cancer cells and specific ligands deposited on sensor chips using an IBIS MX96 SPR imager (SPRi). As a model system, cells from the breast cancer cell line HS578T, SKBR3 and MCF7 were used. SPRi responses to Epithelial Cell Adhesion Molecule (EpCAM) antibody and other ligands coated on the sensor chips were measured. SPR curves show a response attributable to the sedimentation of the cells and a specific binding response on top of the initial response, the magnitude of which is dependent on the ligand density and the cell type used. Comparison of SPRi with flow cytometry showed similar EpCAM expression on MCF7, SKBR3 and HS578T cells.



## 1. Introduction

Surface Plasmon Resonance (SPR) can measure real-time biomolecular interactions in the evanescent field of the sensor surface (Schasfoort et al., 2008). SPR is frequently used to provide insight into affinities of interactions between antibodies and their targets without the need to label the biomolecules with a reporter molecule (Roden and Myszka, 1996; Gutierrez-Gallego et al., 2011). Reports on label free measurements of membrane surface antigens and their ligands measured by SPR do exist (Rich and Myszka, 2000). However label free measurement of the dynamics with the surface receptors on whole living cells are much less commonly reported. For monitoring cell binding to immobilized antibodies the cells should be injected. However most commonly they are pre-incubated on a SPR chip (Gorodkiewicz et al., 2011; Hiragun et al., 2012; Milgram et al., 2012; Robelek and Wegener, 2010; Yanase et al., 2012 , 2007a, 2007b). An explanation for the absence of SPRi measurements with cells is the relatively large size of cells (~10 to 15 μm) as compared to the depth of the evanescent field of SPR (~300 nm) and the fact that the optics of most commercially available SPR equipment with flow cells are situated on top of the fluidics (Gutierrez-Gallego et al., 2011). The latter makes it impossible to measure the interaction between the cell surface and the sensor surface coated with the ligands as cells will settle onto the sensor surface by gravitational force. Most popular SPR instruments such as the BIAcore range (Jason-Moller et al., 2006) use fluidic cartridges with tiny valves for operation and sample injection, which are prone to clogging when injecting a cell suspension. Label free detection of the interaction between specific receptors on the cell surface and their ligand interactions using SPRi would have distinct advantages compared to traditional cell based analysis techniques such as flow cytometry or fluorescence microscopy. Antibodies used in SPRi do not need to be conjugated, which means that the conjugate also cannot influence the activity of the antibody and as such the antigen–antibody interaction more accurately depicts in vivo-like conditions. In addition SPRi has the potential to track a cell population in real time and follow subsequent stimulation steps without the need of stopping the measurement or to take time-lapse samples like it would be needed in flow cytometry (Yanase et al., 2013). In this paper the label free real-time monitoring of injected cells from breast cancer cell lines on top of immobilized antibodies against a Cell surface marker are studied. The SPR responses were evaluated when breast cancer cells expressing different Epithelial Cell Adhesion Molecule (EpCAM) antigen densities were exposed to EpCAM antibody coated sensor surfaces using an SPRi cell analysis protocol (Schasfoort et al., 2013).

## 2. Materials and methods

### 2.1. SPRi

For SPRi measurements the IBIS MX96 was used (IBIS technologies B.V., Enschede, the Netherlands) (Beusink et al., 2008). The IBIS MX96 has the capacity to measure 96 parameters simultaneously in

* Correspondence to: University of Twente, 1Room CR4429, Hallenweg 23, 7522 NH, Enschede, The Netherlands. Tel.: +31 53 489 2456.
*E-mail address:* i.stojanovic@utwente.nl (I. Stojanović).

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a single measurement and uses back and forth flow for minimizing the amount of sample and reagents needed for a measurement. The fluidics have been designed in such a way that the sample does not pass any valve. The images of the chip during the cell sedimentation stage were made using a custom designed image grabber.

### 2.2. CFM Spotter

For spotting ligands on the sensor surfaces the Continuous Flow Microfluidic (CFM) spotter was used (Wasatch microfluidics LLC, Salt Lake City, Utah, USA) (Natarajan et al., 2008). The CFM spotter has the ability to spot up to 48 different ligands onto the sensor chip simultaneously under back and forth confined flow. The confined back and forth flow increases the efficiency of the spotting and avoids the risk of evaporation for contact and non-contact droplet based spotting methods. Additionally the SPR image area of the IBIS MX96 allows to apply a double print for generating 96 ligands if an application would require that number of parameters.

### 2.3. SPR chips

Easy2Spot® pre-activated G-type chips were used (Ssens bv, Enschede, The Netherlands) as gold SPR sensor surfaces. The chips have a 100 nm hydrogel-like gel layer, which enables higher capacity coupling of ligands in the evanescent field. The pre-activated sensors are delivered for easy spotting without the need for ethyl(dimethylaminopropyl) carbodiimide-N-Hydroxysuccinimide (EDC-NHS) activation by the user.

### 2.4. Flow cytometry

For flow cytometric analysis of the cell lines a FacsAria II (Becton, Dickinson, San Jose, CA, USA) was used. To quantify the number of EpCAM antigens on the cell surface the cytometer was calibrated with the QuantiBRITE® PE fluorescence quantification kit (Becton, Dickinson, San Jose, CA, USA).

### 2.5. Antibodies

Unconjugated EpCAM antibody was generated using the VU1D9 hybridoma. Goat anti mouse IgG F(c) antibody was acquired from Rockland immunochemicals inc., (Gilbertville, Pasadena, United States of America). For flow cytometry the EpCAM antibody VU1D9 conjugated to Phycoerythrin (PE) was used.

### 2.6. Cells

Cells from the breast cancer cell lines HS578T (ATCC® HTB-126™), MCF7 (ATCC® HTB-22™) and SKBR3 (ATCC® HTB-30™) were used. The cells were harvested using trypsin and after which they were resuspended in culture medium, washed with PBS and finally resuspended in Phosphate Buffered Saline (PBS) solution containing 0.25% Ethylenediaminetetraacetic acid (EDTA). Cells were used at a concentration of $\sim 2 \times 10^6$ cells per ml.

### 2.7. Chip deactivation buffer

Two chip deactivation buffers were used. The first deactivation buffer was made from a 1% Bovine Serum Albumin solution (BSA) (Sigma-Aldrich chemie GmbH, Steinheim, Germany) in ligand immobilization buffer. The second immobilization buffer was made from a stock solution of 2-aminoethanol (MP Biomedicals LLC, Illkrich, France), it was diluted to create a 100 mM 2-aminoethanol solution with a pH of 8.

### 2.8. Ligand immobilization buffer

A 10 mM solution of immobilization buffer with pH 4.5 was made using anhydrous sodium acetate (Sigma-Aldrich chemie GmbH, Steinheim, Germany) and acetic acid (Merck Schuchardt OHG, Hohenbrunn, Germany). First a 0.2 M stock solution was made of both components, then from these stock solutions 1.93 parts of sodium acetate were mixed with 3.07 parts of acetic acid, finally 95 parts of ultrapure demineralized water were added. The pH was checked and if needed adjusted to pH 4.5.

### 2.9. System buffer

PBS 10X was created in house according to common protocol. EDTA di-sodium salt was acquired from VWR (VWR international bv Amsterdam, the Netherlands). To the 1 × PBS system buffer of the IBIS MX96 0.0003% Tween 20 (Thermo Fischer Scientific PLC, Waltham, Massachusetts, USA) and EDTA at a concentration of 0.25% was (later) added to reduce the adherence of cells to the chips.

### 2.10. Placing regions of interest (ROI's)

After deactivation of a chip and prior to each cell measurement regions of interest (ROI's) were placed over the spotted ligands as those are needed for the software to detect and record the local Surface Plasmon Resonance signal independently in real time. The software is measuring the average SPR signal within those regions of interest and they can be modified in terms of size as is desired by the user in supplemental Fig. 1 we show an example of how regions of interest are placed in the EpCAM density experiments. ROI's are placed both on the specific ligand spots and on the negative control surface on which there was no antibody immobilized (see Fig. 2). In the EpCAM density detection experiments the ROI's were placed on specific ligand spots and on non-specific BSA spots, in addition the size of the ROI was doubled in order to maximize the analysis area.

### 2.11. Cell binding to spots with varying ligand concentrations

A G-type chip was spotted with a serial dilution of anti-EpCAM. The spots were created in the CFM with a ligand concentration of 13, 6 and 3 μg/ml. The antibodies were immobilized in the CFM spotter using the sodium acetate buffer for 60 min. As negative controls for the spotting process blank sodium acetate spots were used. For chip deactivation 100 mM 2-aminoethanol was flown over the chip after loading the sensor into the MX96. Deactivation time was 10 min. To run cell samples on the MX96 system a custom script for cell handling was developed. MCF7 cells were prepared at a concentration of $\sim 2 \times 10^6$ ml$^{-1}$ in PBS. 600 μl of cell sample was pipetted into 600 μl PCR vials (Eppendorf Nederland bv., Nijmegen, The Netherlands) and placed into the sample rack of the MX96. Just before aspiration of the cells, the samples were first mixed automatically by the MX96 to resuspend the cells and prevent cell clumping while awaiting aspiration. The cells were then injected with a speed of 80 μl/s and allowed to associate (also called sedimentation when no flow is applied) for 30 min by stopping the flow followed by a dissociation phase for 30 min under defined flow conditions. At the end of the run, regeneration with pH 2 glycine HCl was performed for 1 min.

### 2.12. Chip optimization

To reduce non-specific sticking of cells to the SPR sensor, the chip deactivation protocol after ligand immobilization in the CFM spotter was changed when compared to more common SPR measurements. A 1% BSA solution in sodium acetate buffer was