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Notes & Tips

Label-free cell profiling

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ABSTRACT

A surface plasmon resonance (SPR) array imaging method is outlined for label-free cell profiling. Red blood cells (RBCs) were injected into a flow chamber on top of a spotted sensor surface. Spots contained antibodies to various RBC membrane antigens. A typical sensorgram showed an initial response corresponding to cell sedimentation (S) followed by a specific upward response (T) corresponding to specific binding of cells during a critical wash step. The full analysis cycle for RBC profiling was less than 6 min. The sensor surface could be regenerated at least 100 times, allowing the determination of a cell surface antigen profile of RBCs.

Studies focusing on retrieving qualitative and quantitative information on cell membrane antigens are relevant for various applications [1,2]. The most widely used technologies for cell surface antigen determination are flow cytometry and fluorescent microscopy. For both techniques, antibodies targeting the cell membrane antigens are fluorescently labeled and the fluorescent signals obtained from the cells correspond to the density of the antigens. To assess multiple antigens simultaneously, antibodies are labeled with different fluorochromes. In this manner, multiplexing of at least six antigens can be performed routinely, a number too low for detailed antigen screening of red blood cells (RBCs)[1]. Multiplex typing is also possible using genotyping approaches; however, those methods are hampered by null alleles, resulting in false-positive results, and are accompanied by relatively high costs, similar to cytometrical methods. Therefore, the inexpensive agglutination-based methods are still being used for RBC typing to ensure safe blood matching between donor and transfusion recipient. Currently, all blood donors in The Netherlands are serologically typed for the AB0, DccEe, and K antigens. A subpopulation is also typed for other clinically relevant antigens (e.g., FY, JK, MNS) to be able to provide fresh antigen-negative blood for individuals who have developed antibodies against those antigens. Agglutination-based detection in cartridges or in tubes for RBC typing is cheap but laborious and is not suitable for multiplexing.

The use of a label-free method such as surface plasmon resonance (SPR) imaging may overcome these issues. Multiplexing can be achieved by spotting the various antibodies on the surface of an SPR imaging sensor at different positions. Previous attempts to achieve this, using either stationary live cells or, as in this report, soluble RBCs, have neither solved how to accomplish massive parallel cellular analysis nor solved how to realize it quickly in a matter of minutes. This report describes a new SPR imaging detection method for typing RBCs using a critical sedimentation followed by a wash step of antibody spots recognizing RBC antigens on a sensor surface. Although label-free analysis of biomolecular interactions by SPR is widely used for determination of binding kinetics [3,4], the label-free binding of cells to ligand-spotted surfaces [5,6] has not been studied intensively for several reasons:

- Suspended cells under certain shear rate conditions are bounced from the wall and will not interact with the sensor surface because of the size of the cell in relation to the thickness of the stagnant layer under laminar flow conditions.
- For practical reasons, most commercial SPR instruments are configured with optics on top of the fluidics to avoid leakage of liquid into the optical compartment of the instrument. In these instruments, cell sedimentation will occur at the surface opposite to that of the SPR sensor.

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[1] *Abbreviations used:* RBC, red blood cell; SPR, surface plasmon resonance; CFM, Continuous Flow Microspotter; HSA, human serum albumin; PBS, phosphate-buffered saline; ROI, region of interest; S, sedimentation; RU, response units; T, total response.

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- Cells and particles clog valves and tiny channels in microfluidic cartridges.
- Cells should first be homogeneously suspended in back-and-forth flow before injection of the cells into the label-free sensing area. Many systems allow one direction of flow only.
- A cell is many times (>20×) larger than the penetration depth of the evanescent field (~0.5×λ of incident light), so only a small part of the cell is within the decaying evanescent sensing field.
- Cell interactions to immobilized antibodies/antigens do not show 1:1 binding kinetics because of the multiple antigens present on a cell. Avidity-based models using serial dilutions of the ligand densities should be applied for the number of interactions and for qualifying the affinity of the cell antigen–antibody interaction.
- Responses due to cell adhesion will depend on the orientation and packing density of bound cells. A nonlinear response is expected close to saturation levels of the surface area.

Here we report a method on how specific interactions of RBCs to an array of anti-RBC antibodies spotted on a sensor can be achieved and used for antigen typing of RBCs—a method that can, in principle, be used for any cellular detection in a label-free manner. We chose to use the IBIS MX96 instrument (IBIS Technologies, Enschede, The Netherlands) because it is capable of simultaneous label-free SPR measurements of 48 to 96 spots and has the resolving power to tackle antigen typing [7]. It contains reversed optics and back-and-forth flow fluidics, allowing for sedimentation and controlled flow mixing of cells. Valve-less injection of samples and wide 1-mm (internal diameter) tubing allow smooth aspiration of cell suspensions without clogging.

As a proof of principle for a multiplex RBC typing on an SPR imaging device, a planar carboxy-modified gold layer sensor without hydrogel (IBIS Technologies) was spotted with IgG monoclonal antibodies specific for RBC antigens using a Continuous Flow Microspotter (CFM, Wasatch Microfluidics, Salt Lake City, UT, USA) [8]. In total, 48 spots were created with ligands of anti-RBC antigen immunoglobulins, including anti-A (mouse IgM, Birma-1, Merck/Millipore, Scotland, UK), anti-M (mouse IgG1, LM140/110, Merck/Millipore), anti-D (human IgG1, 8D8, Sanquin, Amsterdam, The Netherlands), anti-glycophorin A (mouse IgG1, CLB-ery-1, AME1, Sanquin), and reference spots with human serum albumin (HSA, Sigma–Aldrich A8763) and phosphate-buffered saline (PBS). Anti-A recognizes the A antigen, a glycan structure on RBCs. Anti-M recognizes the M-antigen of the MNS blood group system. Anti-D reacts with the rhesus D antigen present on D^+ RBCs and absent on D^- RBCs. Anti-glycophorin A is directed against glycophorin A, the most abundant membrane glycoprotein of the RBCs and the carrier protein of the MNS allo-antigen system. The monoclonals were purified by protein A (GE Healthcare) and rebuffered to a PBS buffer. An optimal coupling concentration and pH was found to be approximately 2 μg/ml in 10 mM acetate at optimal pH (ranging from 5.0 to 6.0), although this varied slightly between different antibodies based on spotting density and responses to antigen-positive versus antigen-negative cells (an example is shown in the Supplementary material). After the spotting process, the sensor was positioned in the IBIS MX96. Samples were exposed to all spots at the same time, and interactions to all 48 spots were monitored in real time. The sensor surface can be used many times by a regeneration process where the RBCs are removed from the covalently coupled ligands, keeping assay costs low. Detailed operations of the predecessor of the IBIS MX96 instrument and the CFM are described elsewhere [7,8].

Prior to injection of the cells, the diluted RBC suspension ($20*10^6$ cells/ml obtained from 5 μl of blood) was both mixed (by repeated aspirating and dispensing) and transported from a microtiter plate (200 μl) using the built-in autosampler by XYZ positioning of the needle via Teflon tubing into the flow chamber. Mixing was necessary to homogenize a pellet of cells in the microtiter plate well. The flow chamber has dimensions of 6 × 10 × 0.2 mm (width × length × height) and contains the array of spotted ligands. A total volume of 800 μl was aspirated into the tubing, and a cell suspension of 200 μl was passed into and through the flow chamber with a flow speed of 120 μl/s. After stopping the flow, a slow accelerated increase of response wasobserved while RBCs sedimented onto the complete sensor surface, including the reference spots. However, after resuming the flow after 3 min of sedimentation at 20 μl/s in the backward direction, unbound cells and superfluous cells were washed from the surface (Fig. 1).

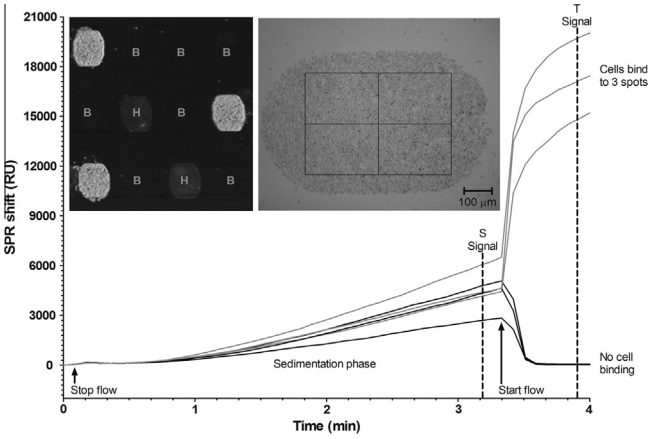

Fig.1. Cellular detection using multiplex SPR. The sensorgram shows binding of RBCs to three specific spots with anti-A and three reference spots without ligands. After stopping the flow, the cells sediment on the sensor surface nonspecifically and a sedimentation signal (S) was observed for all regions of interest (ROIs). After restarting the flow, washing unbound cells, an upward signal (T) was measured for cells that bind specifically to selected spots, whereas control spots return to baseline level (T = 0). The top left inset is an SPR image (reflectivity) of the sensor surface showing three specific anti-A spots with RBCs and some negative spots (B) are visible, whereas the spotted protein (HSA: H) is visible on some spots. The top right inset is a microscopic view of a single spot with four quadrants of ROIs showing M^+ RBCs bound to an anti-M spot. SPR shift in resonance units (RU).

Although the kinetic curves during sedimentation showed a nonexponential binding curve, a delay in response was always observed after stopping the flow. The response then slowly increased on all spots, also negative control spots, in direct relationship with the number of cells that landed on the surface (Fig. 2A and data not shown). The cells landed during the sedimentation phase in a region of interest (ROI) with size smaller than a spot, encompassing as large a surface area of the spot as possible. Therefore, the method monitors the average interaction of all cells that land within the ROI (see Fig. 1 inset), which changed the refractive index in the evanescent field and resulted in a signal increase. This sedimentation (S) response can then be recorded for all spots (Fig. 1). After resuming the flow, washing away unbound cells, a downward signal to the baseline was observed on reference spots, or if the cell was negative for antigen-specific spot, caused by flushing of the sedimented cells. However, an upward signal was observed for spots with antibodies recognizing antigens on the cell surface (Fig. 1). For some spots, the upward signal can be three times the S response (>15,000 resonance units [RU]). We observed some unequal distribution of RBCs over the whole chip, with some spots having less RBC sediment and, therefore, lower S signal. Importantly, the magnitude of the specific total response (T in Fig. 1) was in direct relationship with the sedimentation response (Fig. 2A), allowing for normalization of the data between spots and between experiments ($n > 100$) by expressing the specific response as a ratio T/S (Fig. 2A). Only cells expressing an antigen corresponding to an antibody on a spot (ROI) gave a specific response

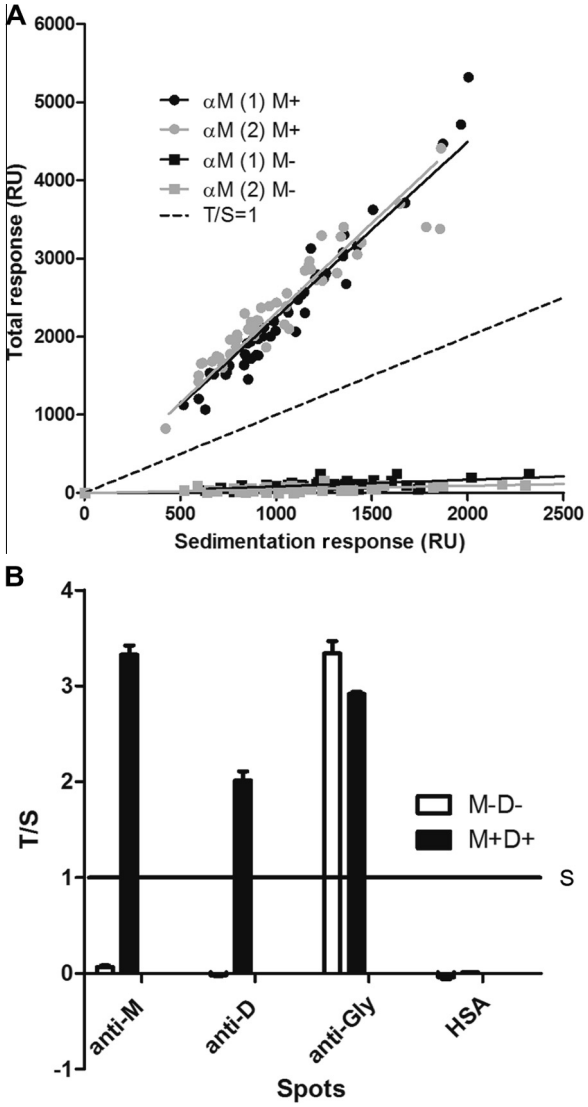

Fig.2. Typing of RBCs on SPR biosensors. (A) SPR sensors (1 and 2) were spotted with anti-M to type RBCs (typed with standard agglutination methods), and the signals ($n > 100$) are measured as explained in Fig. 1. The total response (T) on anti-M spots was plotted against the sedimentation response (S), resulting in a highly significant correlation ($P < 0.0001$) for M^+ cells with respect to M^- cells. Therefore, the response (T/S) was used as a normalized response value. (B) SPR sensors were then spotted with anti-M, anti-rhesus D (anti-D), anti-glycophorin A (anti-Gly, positive control), or HSA (negative control) to type RBCs (typed as M^+D^+ or M^-D^- with standard agglutination methods), and the signals were measured and expressed as normalized T/S, reliably typing the cells as either D or M positive or negative. The columns and error bars represent means and standard errors of means, respectively.

with T/S > 1, allowing for accurate detection of the expressed blood group (Fig. 2B).

From these observations the kinetic process of cell binding has at least two typical features:

- After injecting the cells, a delay of response signal is observed after stopping the flow. This typical delay is observed only when large particles (cells) are applied but never when, for example, an antibody sample is injected.
- Cells that bind specifically to the immobilized ligand molecules will show an upward response, after starting the flow again, whereas a downward response is observed from reference spots or other spots that do not bind cells. If the shear rate is below a certain critical value of disruption, the response is stable and the cells will stay on the spots and no off-rate is observed.

These typical effects can be explained as follows:

- While cells flow in tubing and flow chambers, the laminar flow will deplete the stagnant layer of cells close to the wall. In other words, after a lateral transport of the cell suspension over the sensor surface, cells are not in direct contact with surfaces because of shear forces but will stay in the middle of the stream. Therefore, the red blood cells will first enter the cell-free stagnant layer before settling in the evanescent field of the sensor; hence, the delay of sedimentation response is observed.
- When cells are bound to specific spots of the sensor surface, the flow will induce a reorientation, repacking, or pressing down of the biconcave RBCs to the surface. Energetically, it is more beneficial that cells are pressed closer to the surface induced by the flow. This results in a larger part of the specifically bound cells that is localized in the evanescent field, and an upward response is observed. In contrast, sedimented cells that are not recognized by the coated antibodies (and reference spots) will be washed away, and the response will return to the baseline. However, when the flow is too high (data not shown), the shear forces on the cells will increase as well and the cells will disrupt from the surface. Hence, discrimination can be made between weak/nonbinding cells and specific bound cells by adjusting the flow speed in the backward direction after the sedimentation phase (see Supplementary material).

Label-free cell profiling measurements could be repeated more than 100 times using a single sensor surface (see Supplementary Fig. 1 in supplementary material). The development of an economical high-throughput method for RBC typing has the potential to reduce the hands-on time and costs, provide automation, and increase donor–recipient matching in the general blood bank practice. The method has broader implications for cellular typing in general because it can be extended to other cellular screening and typing applications.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.04.001.

References

[1] J.G. Quinn, R. O'Kennedy, M. Smyth, J. Moulds, T. Frame, Detection of blood group antigens utilising immobilised antibodies and surface plasmon resonance, J. Immunol. Methods 206 (1997) 87–96.
[2] V. Chabot, C.M. Cuerrier, E. Escher, V. Aimez, M. Grandbois, P.G. Charette, Biosensing based on surface plasmon resonance and living cells, Biosens. Bioelectron. 24 (2009) 1667–1673.
[3] W. de Lau, N. Barker, T.Y. Low, B.-K. Koo, V.S.W. Li, H. Teunissen, P. Kujala, A. Haegebarth, P.J. Peters, M. van de Wetering, D.E. Stange, J. van Es, D. Guardavaccaro, R.B.M. Schasfoort, Y. Mohri, K. Nishimori, S. Mohammed, A.J.R. Heck, H. Clevers, Lgr5 homologs associate with Wnt receptors and mediate R-spondin signaling, Nature 476 (2011) 293–297.
[4] R.B.M. Schasfoort, W. de Lau, A. van der Kooi, H. Clevers, G.H.M. Engbers, Method for estimating the single molecular affinity, Anal. Biochem. 421 (2012) 794–796.
[5] J.S. Maltais, J.-B. Denault, L. Gendron, M. Grandbois, Label-free monitoring of apoptosis by surface plasmon resonance detection of morphological changes, Apoptosis 17 (2012) 916–925.
[6] Y. Yanase, H. Suzuki, T. Tsutsui, I. Uechi, T. Hiragun, S. Mihara, M. Hide, Living cell positioning on the surface of gold film for SPR analysis, Biosens. Bioelectron. 23 (2007) 562–567.
[7] A.M.C. Lokate, J.B. Beusink, G.A.J. Besselink, G.J.M. Pruijn, R.B.M. Schasfoort, Biomolecular interaction monitoring of autoantibodies by scanning SPR microarray imaging, J. Am. Chem. Soc. 129 (2007) 14013–14018.
[8] S. Natarajan, P.S. Katsamba, A. Miles, J. Eckman, G.A. Papalia, R.L. Rich, B.K. Gale, D.G. Myszka, Continuous-flow microfluidic printing of proteins for array-based applications including surface plasmon resonance imaging, Anal. Biochem. 373 (2008) 141–146.