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Microarray Optimizations for High-Throughput Glycoprofiling of Cancer Sera

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Abstract

Aberrant glycosylation is one of the characteristic hallmarks of cancer with increased sialylation, increased branched-glycan structures and overexpression of core fucosylation being some of the prominent manifestations. Serum-based biomarkers indicative of cancer are the most desirable form of biomarkers that can be used for personalized daily care in screening, early and rapid diagnosis, establishing prognosis, monitoring treatment, and detecting relapse in cancer patients. Microarrays have been the sole analytical platform, since their development in the past two decades, for analyzing carbohydrate-mediated recognition events in a high-throughput manner. Several factors influence the final readout of these binding events. Here we demonstrate the optimization of one of the key extrinsic parameters in glycoprofiling using glycoprotein-based glycan arrays namely buffer composition and incubation timing. The optimized conditions were then adopted to glycoprofile 28 breast and 16 lung cancer patients' sera.

Keywords: *Microarray, glycoprofiling, high-throughput, lectin, glycan, cancer, diagnostics*

Introduction and Objectives

Glycosylation happens to be a canonical and multifaceted co- and posttranslational modification in eukaryotes. It plays a ubiquitous role in regulating several physiological and pathological functions throughout the lifespan of an organism [1]. Erroneous and defective glycosylation machinery can lead to severe medical consequences and may also prove to be fatal [2]. Anomalies in glycosylation patterns, majority of the times are associated with malfunctioning of the glycosylation machinery which is often a manifestation of pathological condition. Therefore, faulty glycosylation also happens to be an anticipatory outcome of several pathological abnormalities encompassing the likes of diabetes mellitus, autoimmune diseases, anemia and happens to be a classical hallmark of various cancers [3 – 6]. Aberrant glycosylation in cancer demonstrates increased sialylation, increased branched-glycan structures and overexpression of core fucosylation being some of the prominent manifestations.

Serum based biomarkers indicative of cancer are the most desirable form of the biomarkers that can be used for personalized daily care in screening, early and rapid diagnosis, establishing prognosis, monitoring treatment, and detecting relapse in cancer patients.

Considering the colossal diversity demonstrated by glycans, high-throughput methods of glycoprofiling become a mandate for screening aberrancies in glycosylation. Glycans and corresponding carbohydrate-binding proteins or lectins prove to be instrumental in high-throughput glycoprofiling by protein-based glycan arrays. Microarray has been the sole analytical platform, since their development in the past two decades, for analyzing carbohydrate-mediated recognition events in a high-throughput manner [7, 8]. Several factors influence the microarray analyses these carbohydrate-glycan binding proteins (GBPs) [9]. Several factors influence the final readout of these binding events. Here we demonstrate the optimization of one of the key extrinsic parameters in glycoprofiling using glycoprotein-based glycan arrays namely buffer composition and incubation timing. The optimized conditions were then adopted to glycoprofile 28 breast and 16 lung cancer patients' sera.

Materials and methods

All analyzed serum samples were diluted in PBS to the same concentration (100 µg/mL), transferred into the 384-well microtiter plate and spotted to the epoxy microarray slides (NEXTERION Slide E, Schott, Germany) in triplicates or pentaplicates (based on the analysis). Each spot consisted of one (1 drop ≈ 0.5 nL) using a non-contact piezoelectric sciFLEXARRAYER S1 microarray spotter and piezo dispense capillary PDC 80, PDC 90 (type 3, 4) (Scienion AG, Berlin, Germany) at the temperature of source plate of 11 °C and humidity of 55 %. The printing was performed into 8 identical subarrays for buffer optimization experiments as well as cancer sera glycoprofiling. For buffer optimization experiments control serum samples (Thermo Fischer AG) and fetuin were spotted in 5 different concentration (6.25-100µg/ml) and 6 different volume configurations (1-7 drops). The buffer optimization experiments were also performed with 8 different buffer composition (Tab. 1) for blocking the slides. Slides with immobilized samples were incubated in the spotter for 8 hours with humidity increased to 60 %. Prepared slides were stored in the fridge until the next incubation steps. On the day of incubation, slides were taken out of the fridge and left for 30 mins to attain room temperature before the mask was attached. Unreacted epoxy groups were blocked with a solution of 1x Vector Carbo-free Blocking solution for 60 minutes at room temperature with gentle shaking. After washing the slides with PBS containing 0.1 % Tween-20 (PBST), 18 biotinylated lectins (purchased from Vector, Burlingame, USA, with exceptions of rPhoSL and rHEL which were kind gift from Dr. S. Kim, KRIBB, Jeonbuk, Korea) at concentrations of 25 µg/mL in PBST were loaded into 8 subarrays for 60 minutes at room temperature with constant shaking. The slides were washed again with PBST and streptavidin-conjugated with a

fluorescent dye CF647 (Biotium, Hayward, USA) 0,5 µg/mL in PBST was loaded into 8 subarrays for 60 minutes at room temperature. The slides were thoroughly washed with PBST and distilled water, and the residual water was removed by centrifugation. Fluorescent signals were detected using InnoScan®710 fluorescent microarray scanner (Innopsys, Carbonne, France) at the wavelength of 635 nm with laser. The signals were analyzed by Mapix® software (Innopsys). The fluorescence of each spot was measured and corrected for the background signal, and the intensity of the specific interaction was expressed in arbitrary fluorescence units.

Results and discussion

Several studies focusing on various intrinsic factors like slide chemistry, linker chemistry, glycan density and presentation and several other aspects, influencing microarray analyses of glycan-lectin interactions have been performed in the past [9-13]. Further, studies analyzing extrinsic factors including various humidity conditions during microarray printing and buffer compositions have also been reported [14]. However, the influence of blocking buffer composition on the results of glycan and lectin arrays have been assumed to minimal or negligible [9]. Here we performed the blocking of epoxide coated slides printed with fetuin and control serum with different buffer composition as indicated in Tab. 1.

Tab. 1. Buffer Compositions Adopted – 8 different buffer combinations with water, bovine serum albumin (BSA), Tween 20 (T), phosphate buffered saline (PBS) and ethanolamine (Et-OH) were used.

	Buffer Composition	Buffer pH
Buffer 1	H_2O + 3 % BSA	7.03
Buffer 2	H_2O + 0.05 % T + 3 % BSA	7.03
Buffer 3	PBS + 3 % BSA	7.4
Buffer 4	PBS + 0.05 %T + 3 % BSA	7.4
Buffer 5	1X Vector	8.2
Buffer 6	Et-OH	Adjusted to 8
Buffer 7	Et-OH + 3 % BSA	Adjusted to 8
Buffer 8	Et-OH + 0.05 % T + 3 % BSA	Adjusted to 8

Based on the scanning measurements buffer 7 and 8 i.e., Et-OH with BSA showed the highest background signal on the epoxide coated slide surface. Buffer 1, 2 composed of water with BSA in presence and absence of tween also showed considerable degree of background signal. Buffer 3, 4 and 5 showed the least background noise signals in the descending order of 3 > 5 > 4. The unusually high signal in case of buffer 1, 2, 7 and 8 might be due to residual

BSA on the slide despite performing washing step with detergent (Tween 20). Regardless the buffer 4 showing the lowest background signal (Fig. 1A), we decided to continue with the commercially available buffer to perform blocking especially considering lower buffer composition variability as against our inhouse recipe of buffer 4. Shortlisting buffer 5 we further performed blocking time optimization at four different intervals of 45, 60, 90 and 150 minutes (Fig. 1B**).** Based on this we couldn't see any further decrease in the background intensities beyond 60 minutes and fixed our blocking time as 60 minutes. This demonstrated that blocking for 60 minutes with buffer 5 efficiently neutralized and cap all the unoccupied epoxide groups on the slide beyond which elaborated blocking duration had no influence in further reducing the background signal.

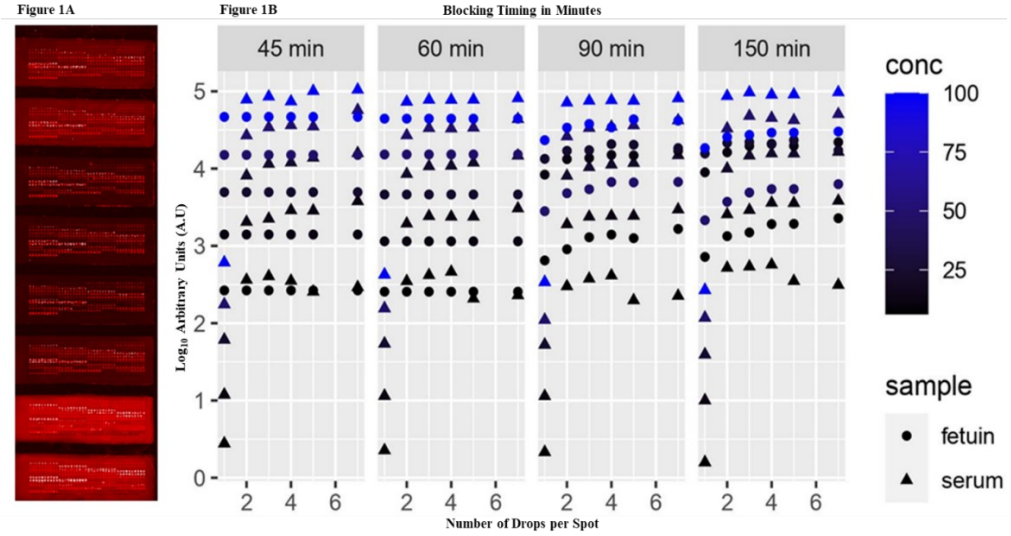

Fig. 1. Blocking Time Optimization – A) Scan image showing background intensities of respective buffers used for blocking; B) Plot showing background intensities of various drop and concentration combinations.

Adopting these blocking optimizations, we performed glycoprofiling of sera samples from patients with lung and breast cancer with a panel of 18 lectins with different sugar specificity. In the Fig. 2 are shown the results, and as it can be seen, a combination of α-galactose binding lectin GSL I-B4 (*Griffonia Simplicifolia* lectin I isolectin B4) and sialic acid specific lectin SNA (*Sambucus nigra* lectin) can help to detect and distinguish aberrant glycosylation patterns amongst these two types of cancer samples. Further microarray analyses with more lectins, mass spectrometric analyses to determine the definite glycan structures and statistical evaluations need to be performed to have definite set of lectins which can efficiently detect aberrant glycosylation patterns in these types of cancers and further aid in diagnosis and biomarker discovery.

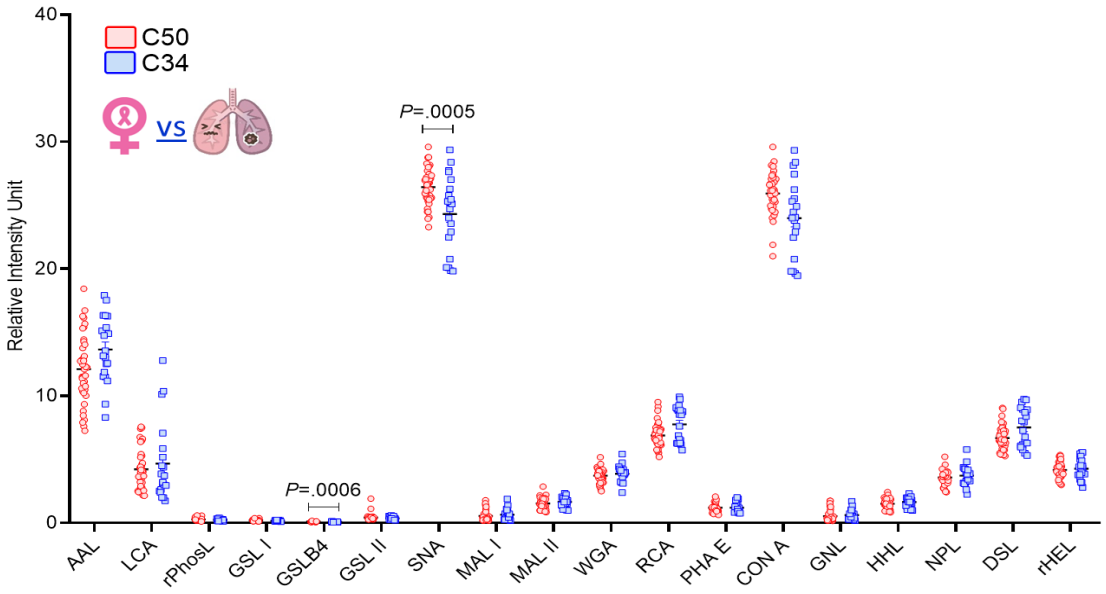

Fig. 2. Lectin-based glycoprotein microarray analyses of breast (red circles) *vs.* lung (blue squares) cancer sera – Signal intensities of interactions with lectins are in relative units. Statistically significant differences are shown for lectins with p-values < 0.001 (t-test).

Conclusion

This study demonstrates that optimization of blocking buffer composition and blocking duration depending on the slide surface chemistry can minimize background noise and enhance throughput while glycoprofiling using microarray. Further optimization studies analyzing both intrinsic, extrinsic factors as well as glycan/lectin array data analysis and data presentation will aid in devising a toolkit for microarray analyses and reducing lab-to-lab variations. Such optimizations can become a part standard operating procedure while designing microarray studies for glycoprofiling and can thereby enhance the efficiency of this platform and better apprehension of the glycoprofiling performed. The optimized conditions were applied to glycoprofile 28 breast and 16 lung cancer patients' sera, and glycoprofiling of next more than 200 cancer patients' sera with different types of cancer is in progress.

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