

Comparison of Slide Surfaces for the Fabrication of Pain-Related Message Molecule Antibody Microarray

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Current therapeutic principles are step-by-step trials with multiple drugs for pathological pain. Such treatment plans often make physicians or patients uncertain and discouraged. The real problem is focused on the limited view of pain network. It was impossible for past researchers to observe so multiplexed proteins changes within different pain syndromes. Conventional methods for protein expression focus only on one or a few targets. However, the investigation of new therapeutic targets to pain needs a better realization of the global regulation network. Fortunately, with advancement of new technologies, it is possible to able to examine hundreds and thousands of protein simultaneously right now. Such large-scale studies have the promise to assemble individual pieces together to gain insights into the overall picture of proteome-wide modifications on pain mechanisms.

Antibody microarrays are an emerging technology that promises to be a powerful tool. It has significant applications in basic and clinical researches, particularly because of the rapidity of the experiments. Such analysis is possible, if many experiments with highly parallel with quantitative information can be performed. Especially for clinical researches, the results from antibody microarray could be correlated with clinical information to assess the clinical value of multiple proteins or sets of proteins. The microarray format facilitates not only the rapid evaluation of many proteins individually but also the evaluation of coordinate patterns of expression. Therefore, we tested four commercial antibody microarray surfaces printing on substance P (SP), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), tumor necrosis factor- α (TNF- α). To improve the performance, protein G coating method was introduced into slide surface. The results showed the best performance can be achieved in aldehyde-derivatized slide by protein G coating method.

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Introduction

Long-term pains are complex diseases that cause significant disruption patients' life quality, such as sleep disturbances, decreased functional capacity, depression, and social financial burden. Comorbidities with depression and other chronic illnesses contribute to a decrease in quality of life¹. The underlying molecular mechanisms are still not completely understood, and as a consequence, treatment is unsatisfactory in many cases². Though the pain mechanisms have been studied extensively, no one representative and integrated theory is produced. Past pain researches were only to focus and clarify some part of complicated relationships. It is very possible that the exact mechanism is too intricate to explain with simplified or one-way cause-and-effect theory. In the view of systems biology, the pathological pain owns the robust characteristics and defends outer perturbations. Robustness is a system-level phenomenon to maintain the systematic homeostasis. The individual components of a system may be robust themselves. The robustness is a property of the assembly of components and it can't be fully understood by examining them individually. Thus, the robust system of pathological should pain carry on with its abnormal pain perception despite perturbations (internal or external), unpredictable environments (drug-intake), and unreliable components³.

Molecular targets associated with diverse pain conditions may regard as diagnostic markers. Determining pain-related molecular targets may help to elucidate molecular mechanisms underlying pathogenesis and pathophysiology of pain. Another clinical importance is the need to identify potential therapeutic targets. Conventional methods for protein expression focus only on one or a few targets. With

advancement of new technologies, hundreds and thousands of protein can be examined simultaneously now. Such large-scale studies have the promise to assemble individual pieces together to gain insights into the overall picture of proteome-wide modifications. In order to detect low-abundant molecular targets and observe the dedicated changes of each component, high sensitive proteomic techniques should be applied for identification and quantitation.

Antibody-based microarray have emerged as a strong candidate and complementary proteomic technology, providing the means to perform high multiplexed, rapid, selective and sensitive profiling of even non-fractionated, directly labeled complex proteomes^{4,7}. A major advantage of the antibody microarray, compared with conventional protein assays such as western blotting and ELISA, is that rather than comparing one or a few proteins, and one experiment can examine hundreds of proteins at one time. Unlike 2D gel electrophoresis, a single antibody microarray can measure the expression of both small- and large-molecular-weight proteins, regardless of their isoelectric points. Miniaturized microarrays ($<1\text{cm}^2$) can be printed with numerous individual antibodies (<2000 antibodies/ cm^2) in discrete positions (about $200\text{-}\mu\text{m}$ -sized spots) in an ordered pattern. The antibody microarray is then incubated with few amounts (μL scale) of labeled clinical samples, where later specifically bound analytes are detected and quantified. The read-out generates semi-quantitative microarray images that can be converted into protein expression profiles, revealing the detailed composition of specimens from patients⁷.

For exploring the more extended surveying view, antibody microarray will be one excellent tool for

proteomics of pain research. Due to new application of antibody microarray in pain, we have to do the feasibility study before examination of specimens. Therefore, we evaluated four commercially available slides with different surface modifications and reactive chemistries, such as aldehyde-derivatized slide, FullMoon slide, FAST slide, and MaxiSorp microarray slide. Finally, we can compare one best commercial array surface for further studies of more pain-related message molecules. Then with the same model, the number of target proteins increase and the pain network structure will be closer to completeness. With differential protein expression in neural secretomes, profiling efforts directed towards comprehensive understanding the network of pathological pain, early and improved diagnostics, patient stratification, prediction of relapses, monitoring drug efficacy and so on.

Materials and Methods

Reagents

Bovine serum Albumin (BSA) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). NaCl, Tris (base) and HCl were purchased from J.T. Baker. (Phillipsburg, NJ, USA). Phosphate buffer saline was purchased from One-Star Biotechnology. (Taipei, Taiwan). Glycerol was purchased from Merck (Darmstadt, Germany). Recombinant protein G was purchased from BioVision. (Mountain View, USA). DyLight 649 NHS Ester, ZebaTM Desalt Spin Columns (0.5 ml), and Sodium Borate buffer, pH 8.5 were purchased from Pierce (Rockford, USA). Antibodies against brain-derived neurotrophic factor (BDNF)

(monoclonal, 16.6 μ g/ml), nerve growth factor (NGF) (Rabbit polyclonal, whole antiserum), tumor necrosis factor- α (TNF- α) (monoclonal, 50 μ g/ml), and substance P (SP) (monoclonal, 100 μ g/ml) were purchased from Abcam (Cambridge, UK).

Antibody chip printing

Based on the spots' layout on the chip, four chosen antibodies were aliquoted into 384 wells Polypropylene Microtiter plates (Nunc, Rochester, USA) to make each well contain 10 μ L of antibody by hand pipetting at 4°C. Chosen antibodies were printed onto glass or polymer slides. FullMoon slides (3D porous surface slides) was purchased from Full Moon BioSystems (Sunnyvale, CA, USA), aldehyde-derivatized slide was provided by BaiO (Shanghai, China). After printed these two kind of slides directly by manual slide arrayer (V&P Scientific, Inc., USA), we put printed slides in 4°C refrigerator overnight for immobilization. For the experiments of protein G coating, FullMoon slides, aldehyde-derivatized glass slides, FAST slides purchased from Whatman (Florham Park, NJ, USA), and MaxiSorp microarray slides provided by Nunc (Rochester, NJ, USA) were immersed in 20 μ g/ml protein G in phosphate buffer saline (KH_2PO_4 0.24 g/L, Na_2HPO_4 1.44 g/L, NaCl 8 g/L, KCl 0.2 g/L, pH 7.4) with 1 mM EDTA at 4°C for at least 8 hr. These slides were blocked with 1% BSA at room temperature for 1 hr followed by washing slides with TBST. Later, slides were rinsed by water followed by spun dry and ready for antibody printing. Antibody printing was held by manual slide arrayer. In order to immobilize the spotted antibodies, the slides were put in the 4°C refrigerator for two hours after antibody printing.

Fluorescent Sample Labeling

Among four chosen pain-related proteins, BDNF, NGF, and TNF- α were purchased from Abcam. SP was provided by GeneScript (Piscataway, NJ, USA). We labeled protein sample with DyLight 649 NHS ester with 1:10 molar ratio in 50mM Sodium Borate buffer, pH 8.5 at room temperature for 1 hr. Then, we quenched labeling step by adding the same volume of 3% of BSA in 2M Tris-HCl and shaking at room temperature for one hour. Then, we applied the samples to Zeba™ Desalt Spin columns to remove the excessive dye.

Chip assays for specific binding

We assembled slides immersing protein G or not with ProPlate frame (Grace Bio-Labs, USA) followed by blocking with 1 % BSA at room temperature for one hour. Then, we added sample to each frame to run the chip assay at room temperature for one hour. After washing the frame area on the chip with 250mL TBST (25mM Tris, 140 mM NaCl, 0.05% Tween, pH 7.5) and repeating three times, we dissembled the frame from the chip and then washed chip in the TBST at room temperature for 10 min. Finally, we washed the slides with distilled water at room temperature for 10 min and repeat 3 times, spun dry, then scanned slides by Genepix 4000x (Molecular Devices, CA, USA).

Results

We evaluated four commercially available slides with different surface modifications and reactive chemistries, such as aldehyde-derivatized slide, FullMoon slide, FAST slide, and MaxiSorp microarray slide. The main reason for comparing these slides was to choose one slide with good performance for pain-related message molecules and with no requirement for modifying antibody with some affinity tags before

printing. It was convenient to use for multiple assays, especially for screening high numbers of targets at one time in next study.

We first printed anti-BDNF, anti-NGF, and anti-TNF- α with concentration of 1 μ g/mL, 1:30 dilution solution (polyclonal rabbit antiserum), and 3.3 μ g/mL printed concentration onto each slide, respectively. Afterwards, three pain-related message molecules antibodies were immobilized on Fullmoon and Aldehyde slides with three identical spots per slide. Three particular antigens, BDNF (2.5 μ g/mL), NGF (1.5 μ g/mL), TNF- α (12.5 μ g/mL) were tested in different slide surface individually. Aldehyde-derivatized slide performed slightly better than three other slides (Fig.1). In aldehyde-derivatized group (Fig.1 (a)-(c)), NGF had the strongest signal intensity within three pain-related message molecules, but the non-specific binding in other antigens existed in the same time. In Fullmoon group (Fig.1 (d)-(f)), none of these message molecules can be revealed, and no other non-specific binding was noted. Our preliminary data cannot meet our expectation of chip performance. Hence, for the better chip performance, we first introduced protein G coating fluorescence-based antibody microarray without adding any affinity tag into antibodies.

Meanwhile, we printed anti-SP antibody (2 μ g/mL) on each slide additionally and tested other slides such as FAST and MaxiSorp slides in the following experiments. All tested array surfaces were coated with protein G, unified the orientation of IgG. In the aid of protein G, we got much better performance in aldehyde-derivatized group and FAST group (Fig.2). In the other two groups, no any detection signal was found. Between the aldehyde-derivatized group and FAST group, the NGF

signal was stronger in the former than in the latter. In the aldehyde-derivatized array, no nonspecific binding was showed. In general, compared with the performance between two slide surfaces, the aldehyde-derivatized group was better than FAST group and more suitable for NGF detection.

However, other pain-related message molecules still were detected in this condition, even with protein G coating. Furthermore, after raising up the concentration of anti-BDNF, anti TNF- α to 16.6 μ g/ml, 20 μ g/ml, we got strong signals in BDNF and TNF- α antibody on aldehyde slide (Fig.3). Unfortunately, SP was not revealed one good signal through increasing concentration of spotting antibody (data not shown). An overview of these slide characteristics and performances were shown in Table 1.

Fig. 1.

Array with DyLight 649 NHS ester fluorescence scanned image (Cy5 scanner channel) showed that the spot morphology for anti- BDNF, anti-NGF, and anti-TNF- α antibodies were printed onto aldehyde by treating with BDNF, NGF, and TNF- α . Similarly, Fullmoon surfaces were incubated with BDNF, NGF, and TNF- α . Each experiment was performed with slides that were printed and processed on different days. Every white frame in array image indicated the specific antibody sites for identical antigens

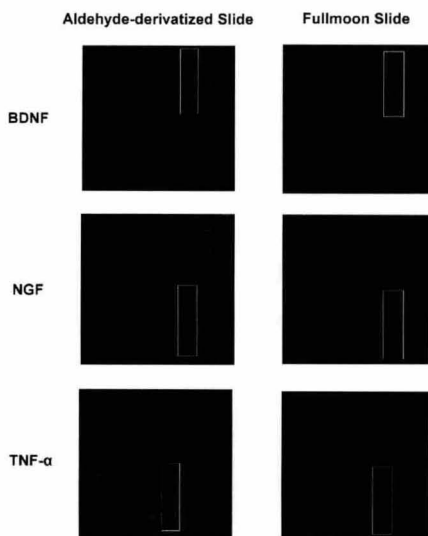


Fig. 2.

Array with DyLight 649 NHS ester fluorescence scanned image (Cy5 scanner channel) showed that the spot morphology for anti-BDNF, anti-NGF, anti-TNF- α , and anti-SP antibodies were printed onto aldehyde, Fullmoon, FAST, and Maxisorp slides coating by Protein G and treating with different specific antigens: BDNF, NGF, TNF- α , and SP. Each experiment was performed with slides that were printed and processed on different days. Every white frame in array image indicated the specific antibody sites for identical antigens.

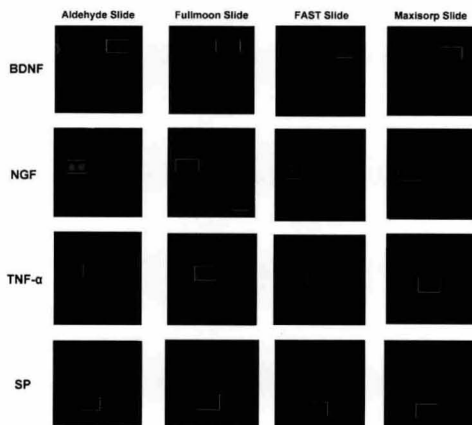
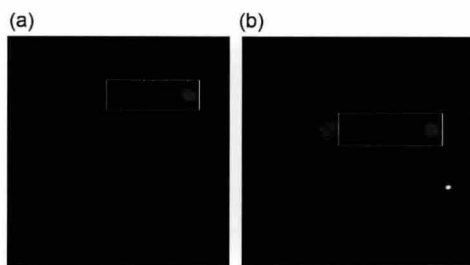


Fig. 3.

Array with DyLight 649 NHS ester fluorescence scanned image (Cy5 scanner channel) showed that the spot morphology for higher (a) anti-BDNF, and (b) anti-TNF- α printed concentration, were printed onto aldehyde slides coating by Protein G with different specific antigens: BDNF, and TNF- α . Each experiment was performed with slides that were printed and processed on different days.



Discussion

Antibody microarray slide surfaces are designed for preventing droplet solution from spreading, limiting spot size, unifying spot morphology, and condensing

proteins in printed arrays. Good spot morphology represents not only as identical printing conditions, but as a uniform quality in this array surface. In our study, one antibody per protein target was designed, and thus direct labeling strategy is easier to conduct the assays, as opposed to sandwich assays and competitive assays. Since cross-reactivity between capture antibodies is not worried, the method will be simple and practical⁸. Furthermore, test samples can be mixed with others samples and the experiments can be under the same condition without considering inter-experimental bias⁹. Hence, we chose the directly labeling method with DyLight 649 NHS ester by linking the primary amine group on the samples. This approach also is effective to simplify the procedure as well as reduce the antibody wasting.

According to identity of binding force, the methods of immobilizing antibodies are divided into two parts: noncovalent and covalent interacted fashions. Noncovalent attachment of proteins on the surface may be associated with positively charged (e.g., poly-L-lysine and aminosilane), hydrophobic effects (e.g., nitrocellulose), hydrophilic polyacrylamide¹⁰, or hydrogen bonds. In our results, the FAST and MaxiSorp microarray slide are classified into this group. The former was coated with nitrocellulose and the latter was associated with specific molecules with noncovalent linkage. Because noncovalent binding is regarded as one weak linkage, there is potential for exchange of the capture antibodies or the blocking agents¹¹. It would increase slide noise due to easy exchanges of attached molecules by noncovalent attachment. In the meantime, with lower density of immobilized antibody, it results in lower signal intensities and higher background noise, and thus leads to poor sensitivity and specificity. The

FAST slide surface only preserved weak NGF signal intensity and MaxiSorp slide was kept weak BDNF signal detection. Others array spots were not seen through regular processing of fixation or washing. These two slide surface with poor ability of immobilizing antibodies may be resulted from the nature of noncovalent binding.

By contrast, covalent attachment is mediated with a variety of chemically activated surfaces (e.g., aldehyde, epoxides, and NHS esters) that are highly reactive to amino, thiol and hydroxyl group of proteins, respectively¹⁰. Aldehyde-derivatized slide and FullMoon slide belonged to this class. The former was only plated with aldehyde-derivatized compounds but the latter was layered with one thin-film proprietary polymer that contains various reactive sites, such as amines, epoxides, aldehydes, and NHS esters. Our experimental results showed aldehyde-derivatized slide had signal detection in three of four antibodies, but only BDNF was able to be detected on FullMoon slide. With the poor performance of these commercial slide surfaces, low abundance of pain-related message molecules would not be detected. As we seen, the binding strength of immobilized antibodies was higher in covalent bonds. However, the signal was not proportionally improved. Although antibodies fixed by covalent bonds are not susceptible to exchange, it is possible to reduce activity due to the covalent interactions with paratopes of antibodies¹². In detail, random arrangement of antibodies is likely to lose antigen binding capacity due to covalent linkage of the antigen recognizing sites on slide surface. Therefore, capture antibody has to be organized with more uniform orientation and the signal detection can be more sensitive. If the IgGs are chemically immobilized on the array surface with random orientation, the Fab

part may be buried on the slide. The opportunity of antibody and antigen binding will be reduced. The biological activity of antibody results in irreversibly losing¹³. Protein G, one cell wall protein found in most species of Streptococcal bacteria, was used for coating on slide surfaces in our experiments. Since protein G has one special interaction with Fc fragment of antibodies, the Fab of antibodies can turn upward with Fc part based on protein G¹⁴. The immobilization of IgG by protein G is likely to be the most successful and convenient approach for obtaining a well-ordered orientation¹⁵. In our experiments, we did observe this tendency of improving signal intensity with protein G coating surface. Hence, for detecting low abundant pain-related message molecules, protein G coating array surface is a simple and efficient method without excess processing steps.

Conclusion

In conclusion, we examined four commercial antibody microarray surfaces, including aldehyde-derivatized, Fullmoon, FAST and MaxiSorp slide. Four different pain-related message molecules (BDNF, NGF, TNF- α , and SP) were tested in antibody microarray printing four specific antibodies. Although following the user guides, the performance was good enough for clinical examination. We tried to introduce the protein G coating method for improving detection performance. Compared to four slide surfaces, aldehyde-derivatized slide has the best sensitivity and specificity. If expanding the detecting targets, it will be the choice of four commercial antibody microarray surfaces.

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製造疼痛蛋白質微陣列晶片表面比較之研究

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目前在治療病態性疼痛的原則仍以階段性治療為主，所得到的療效卻是不穩定且無法令人滿意的。真正原因是在於沒有全盤瞭解疼痛。以前受限於研究工具，只能分析某些蛋白質的變化，對於複雜的蛋白質體的波動，則是束手無策。然而，想要有所突破非得從完整的調控網路著手。幸好，在科技進步之下，已有新的技術可以同時分析上百上千種蛋白質，藉由這樣的工具，我們就可以宏觀的蛋白質體的角度，觀察真正疼痛的機制。

而抗體微陣列晶片正是這樣有力的工具。因為它的快速，已經開始運用在基礎及臨床的研究上。同時它也能夠大量平行分析且進行定量的觀察，對於臨床研究上，尤其可以搭配臨床數據，創造出卓越的研究價值。所以，它的成效不只在快速檢驗個別蛋白質的變化，最重要的是可以建構調控路徑。因此，我們在實驗中檢驗了四種常見不同表面的抗體微陣列晶片，搭配了四種疼痛傳遞因子（SP, BDNF, NGF, 及TNF- α ）。同時為了改善效能，我們將蛋白質G被覆在晶片表面。最終結果顯示醛基晶片加上蛋白質G最適合從事疼痛蛋白分析所需。

關鍵語：疼痛，蛋白質G，微陣列表面，抗體微陣列晶片

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