

Profiling of ligand-receptor induced signalling- a novel protein chip technique

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ABSTRACT

Cellular signalling pathways are the master controls of the biology of the cell, which includes cell communication, growth, death, and differentiation. The activities of these signalling proteins directly influence gene function by regulation of the signalling pathways that mediate cellular responses. Recent advanced techniques have given rise to a number of emerging tools for the analysis of cellular signalling that profile the proteome or the protein complement of the genome. However, these tools for signal profiling still face significant challenges such as sensitivity, specificity and be a high throughput method before they are widely adopted. Sensitivity issues are paramount in detecting signalling proteins that are normally in low amounts. Conventional protein chip technology promises to be a powerful tool for large scale high-throughput proteome profiling but there are still significant drawbacks.

Here we report the development and application of a novel multiplexed and high-throughput platform for the quantitative profiling of activated intracellular signalling proteins subsequent to ligand-receptor induced signalling. This spatially addressable biochip platform will allow comprehensive mapping of interconnected signal pathways, through identification of key functional signalling proteins ('nodes') in each pathway and quantifying their state of activity.

Index Term: Antibody microarray, Cell signalling, Protein chip, Proteomics

I. BACKGROUND

Currently, proteome analysis faces a number of challenges which include specificity, sensitivity, quantifiability and the adaptability for high-throughput studies. Though the current conventional method for global elucidation of protein expression patterns is two-dimensional gel electrophoresis, the procedure is laborious, expensive and has poor reproducibility. The detection thresholds of gel staining methods allows detection of only a fraction of the proteome pool and low abundance proteins are often masked by the presence of other proteins present in abundance. 2D gels and SELDI are complementary techniques as more than two dimensions are used to separate the proteins; however both are not apt for global protein profiling as mass spectrometry requires prior protein separation for low abundance proteins (1).

While protein microarray technology has been successful in demonstrating its usefulness for large scale high-throughput proteome profiling (2-7), performance of antibody/antigen microarrays has been only moderately productive (1). Immobilization of either the capture antibodies or the protein samples on solid supports has drawbacks. Denaturation of the immobilized proteins as well as inconsistent orientation of antibodies/ligands on the arrays can lead to erroneous results (8-10). Recently, a new method adopting mass spectrometry techniques has been developed as an alternative to protein microarrays (11), but is time consuming and can be used only for a small set of proteins.

Here we report a multiplexed, sensitive and high-throughput platform for the screening and quantification of intracellular signalling proteins from a complex mixture of proteins. Each signalling protein to be monitored has its capture moiety (specific antibody) linked to a specific oligo 'tag'. The array involves the

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oligonucleotide hybridization-directed (12, 13) localization and identification of different signalling proteins simultaneously in a rapid and easy manner.

In order to accurately quantitate signalling events, the measurement platform should address three key issues: target specificity, target detection and high throughput multiplex detection of the analytes. Antibodies serve as powerful molecular recognition tools in addressing target specificity and hence will be used as the primary capture moiety. Fig. 1 below shows the schematics of the proposed antibody based capture moiety on to a spatially addressable array. The solid support for the immobilized oligonucleotide probes directly

influences the detection limit of the array. An ideal surface has good spot homogeneity with increased sensitivity and good accessibility to the immobilized probes for hybridization (15). A high binding capacity can be achieved by adopting the pseudo-three dimensional dendrimeric linker system (14, 15) to provide a greater surface coverage of reactive sites for covalent probe immobilization.

The proof of concept of this platform as a feasible signalling profiling tool has been reported in this paper.

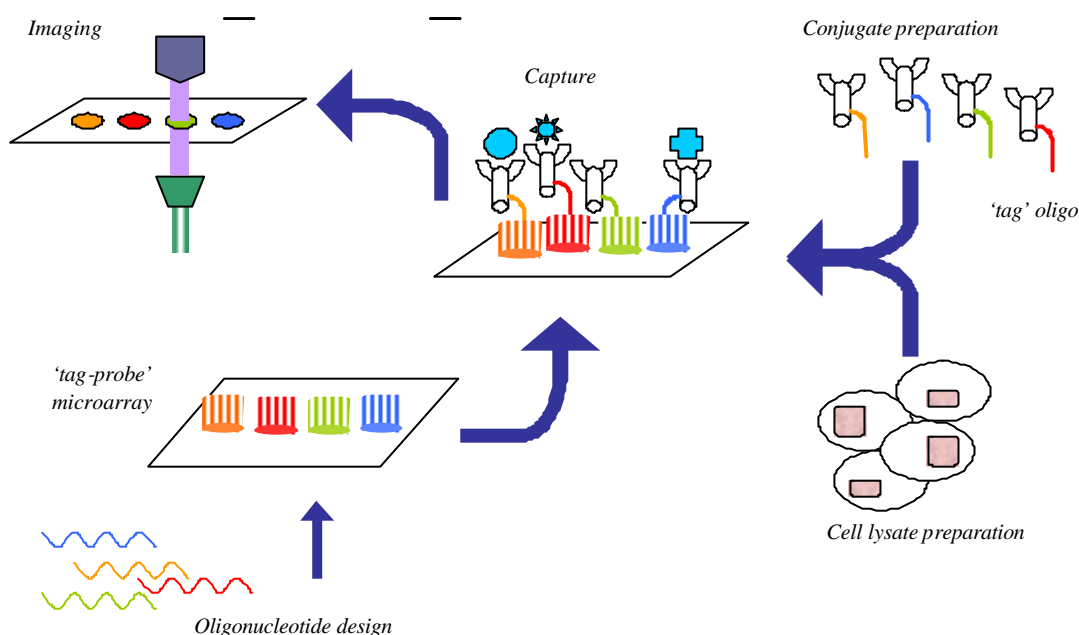


Fig. 1 Schematics of the 'signalling chip' platform

II. MATERIALS AND METHODS

A. Equipment.

GenePix 4000B (Axon, CA) slide laser scanning system, Fluostar fluorescence spectrophotometer (BMG Technologies), MicroCaster™ Arrayer.

B. Preparation of the glass slide with Immobilized Address (Tag-probe oligos).

A reactive surface was created to enable covalent linking of the probe oligos on to a solid support, with high binding capacity. The preparation of the solid support involves the following steps:

- (a) Cleaning of glass slides
- (b) Reactive group functionalisation

Glass slides containing immobilised 20bp poly A and poly T oligonucleotides were made using the MicroCaster Arrayer. This manual method of arraying produces array spots sizes of approximately 600 µm.

C. Preparation of Antibody-oligo conjugates.

The antibody-oligonucleotide conjugates were prepared by covalently linking them via a crosslinker molecule. This hetero-functional crosslinker prevents homo-linkage of DNA and protein molecules.

Monoclonal antibodies (p44/42 MAPK-IgG, Cell Signaling, NEB) were cross-linked to 20bp poly A oligonucleotides. The antibody was purified to remove excess crosslinker and unlinked

oligonucleotides by filtration using membrane spin filters (Microcon).

D. Preparation of labeled cell lysate.

Protein extraction from cell culture.

Neuro 2A cells transfected with GFR1a (gdnf family receptor 1a isoform) were grown overnight in DMEM with 10% FCS, 0.4mg/ml G418 antibiotic. The cells were treated with DMEM containing 0.5% FCS before stimulation.

The sorbitol treated (osmotically stressed) cells were lysed using a mild detergent lysis solution containing phosphatase inhibitors. The lysates were centrifuged at high speed (16,000 rcf) and the supernatant was collected.

Labeling of proteins extracted.

The cell lysates were labeled with Cy3 or Cy5 fluorescent mono-reactive dyes (Amersham Biosciences) in 0.1M sodium bicarbonate buffer. Unbound dye was removed from the labelled cell lysates using protein desalting columns (Pierce).

E. Screening protein profiles on the tag-probe array.

The prepared tag-probe oligo arrays were preblocked to prevent non-specific binding of the labeled cell lysate to the array surface. The antibody conjugate incubated with the labeled cell lysate was allowed to hybridize on the slide in 1×PBS 0.1%TX for 60 min and then washed with 1×PBS 0.1%TX. The hybridized slides were then scanned using the Genepix 4000B (Axon, CA) laser scanner.

III. RESULTS AND DISCUSSION

A. Immobilization of probes on slides

Prepared glass slides were covalently linked to bi-functional Cy3-amino modified 20bp oligonucleotides (in 1X PBS).

A high density of immobilized probes on the glass surface improves the sensitivity of the arrayed spot. The binding capacity of the glass surface to probes was determined using bi-functional NH₂-Cy3 polyT (20bp) oligonucleotides (Proligo) at different reaction concentrations (Fig. 2). A minimum concentration of 50 μ M of oligonucleotides was sufficient to achieve maximal probe density.

B. DNA hybridisation using complimentary oligonucleotides to immobilised probes.

The activities of the immobilized oligonucleotides (Cy3) were measured by hybridising with labeled complementary oligonucleotides (Cy5) (Fig. 3). The resultant yellow colored spot (Fig. 3(b))

indicates the presence of red (Cy5) and green (Cy3) labeled hybridized oligonucleotides.

Non specific interaction with non complementary immobilised probes was similar to background. Furthermore, increasing the density of the immobilised probes did not impair oligonucleotide activities due to steric hindrance.

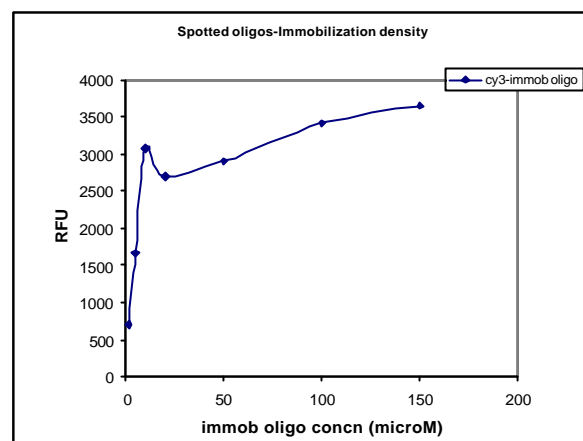


Fig. 2 Binding capacity of the activated support was determined by spotting different concentrations of 5'NH₂-polyT (20bp) Cy3-3' using the MicroCaster™ Arrayer

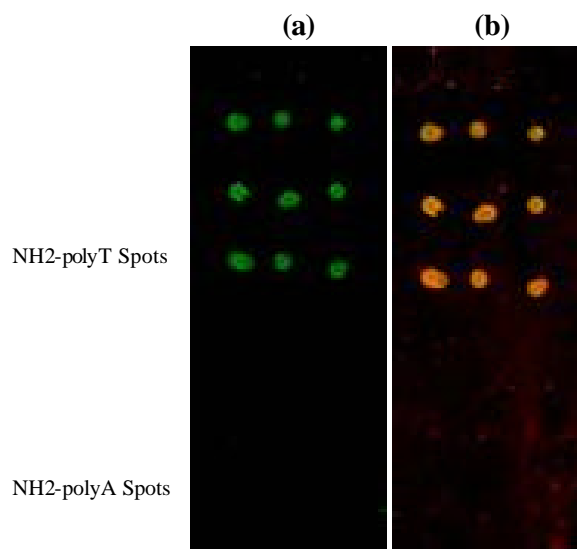


Fig. 3 (a) Fluorescence scanning for the immobilized oligonucleotides on the activated support (b) Detection of Cy5-complementary oligonucleotides hybridized to immobilised oligonucleotides on the same slide.

(a)&(b) spotted NH₂ polyA (20bp) did not hybridize with the Cy5-polyA(20bp) oligos indicating low non-specific hybridization.

C. Antibody-oligonucleotide conjugates preparation.

Crosslinked antibody-oligonucleotide conjugates were prepared using TRITC labeled antibodies and FAM labeled oligonucleotide, to monitor the formation of covalently linked conjugate moieties (Fig. 4).

The results indicates the formation of the capture moiety consisting of oligonucleotides covalently linked to the antibody via the bifunctional cross linker (no cross linker was used for the negative control).

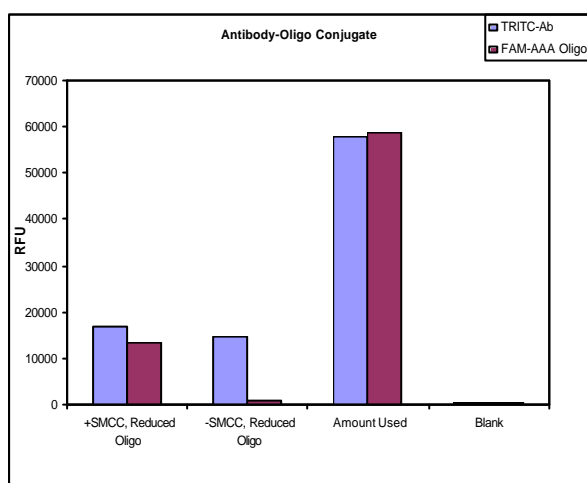


Fig. 4 Antibody-oligonucleotide conjugate formation (capture moiety) was measured by monitoring fluorescence intensities of the TRITC Ab and FAM polyA (20bp).

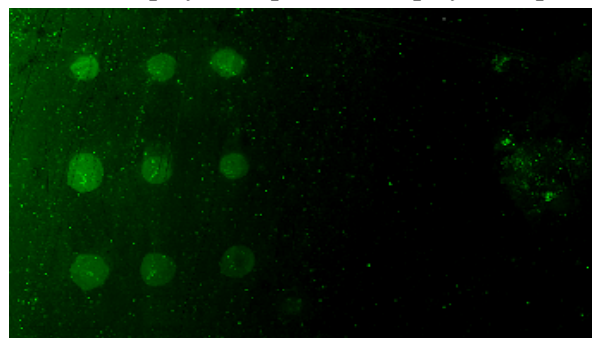
D. Protein capture on the tag-probe array

Monoclonal antibodies (Phospho p44/42 MAPK-IgG, Cell Signaling, Beverly) conjugated to polyA (20bp) oligonucleotides were incubated with Cy3-labeled sorbitol-treated cell lysate. The mixture was then allowed to hybridize to the probe array. Labelled antigen is detected by scanning on the Genepix 4000B laser scanner. Antigen captured by the capture moiety (activated MAPK protein) is indicated by a signal at the target probe spot (Fig. 5a).

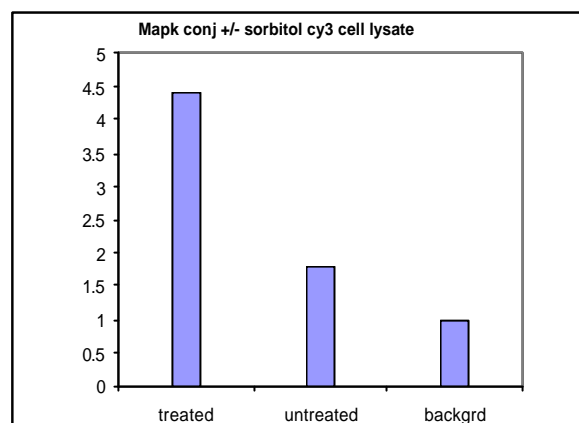
Spots with non complementary sequences to the capture moiety showed signals similar to background (Fig. 5a). Cell lysate proteins did not bind significantly to unspotted areas of the glass slide.

Unstimulated cells similarly assayed resulted in intermediate signals indicating elevated functional protein levels upon stimulation (Fig. 5b).

NH₂-polyT(20bp) NH₂-polyA(20bp)



a PhosphoMAPK Ab-polyA(20bp) conj + Cy3 cell lysate was hybridized onto a slide spotted with NH₂-polyT(20bp) and NH₂-polyA(20bp) (non-specific hybridization control)



b Fluorescence intensity corresponding to the probe spots was measured using the Genepix 4000B laser scanner (PMT 400). Relative protein levels with/without sorbitol stimulation of cells were detected.

Fig. 5 Protein capture on the tag-probe array

IV. CONCLUSION

The platform for protein profiling shown here demonstrates a viable alternative to conventional proteomic tools. The platform is much more rapid than Western blot analysis, the yardstick of protein analysis. It has good specificity with high signal to noise ratios even in the absence of signal amplification as seen in ELISA assays. The microarray format of the platform promises multiplex capabilities and hence high throughput analysis.

Over the past few years there has been tremendous progress in proteomic profiling technology. In an effort to understand the basic events that control complex biological processes, the demand for sensitive miniaturized high-throughput proteomic tools is growing rapidly.

The technology used in the signalling chip described here can be adapted for high throughput screening of protein-protein interactions, protein enzymatic activities, drug discovery as well as clinical diagnostics. Rapid, easy analysis, sample volume economy, and multiplexed high-throughput capability with automation feasibility are the highlights of this method. However there are some limitations. Highly specific antibodies have to be chosen. Oligonucleotide tags for the capture moieties should be designed such that there is no cross homology among the tags.

Increased sensitivity and dynamic range of this technique may be achievable with various other detection methods such as RCA and label-free methods.

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