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REVIEW

Protein microarray: A key approach of proteomics

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Abstract Proteins are vital parts of living organisms, for they consist of the main components of physiological metabolic pathways. The expression and modification status of proteins in cells are time- and space-dependent based upon functions as well as responses to stress. A subject aiming at understanding of protein status under certain physiological condition in a large scale, termed proteomics, is fundamentally important in life science. Although the proteomic investigation mainly relies on electrophoresis, liquid chromatography, and mass spectrometry, protein microarray is emerging as one of new techniques, and its power is being recognized in the field recently. Herein, we highlight progress in protein microarray, both its generation and application.

Keywords proteomics, protein chip, autoantibody, protein interactions, microarray preparation

1 Types of protein microarrays

Generally, protein microarray is a piece of microscopic slide on which different proteins are affixed in an ordered manner. It is divided into three types: forward protein, antibody, and reverse protein microarray. In a forward protein microarray, native or recombinant proteins are immobilized on solid supports; in an antibody microarray, antibodies are immobilized (Lee et al., 2008). The two protein microarrays are also technically termed as the forward phase arrays because the immobilized molecules as baits can capture the analytes. Generally, a forward phase array is incubated with a sample such as a cellular lysate or a serum, and the analyte bindings are examined by fluorescent detection. A reverse protein microarray is made by dotting protein lysates or mixtures in order and specific antibodies are used for screening the antigen distribution in the lysates or mixtures (Spurrier et al., 2008).

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2 Generation of protein microarray

To generate protein microarray, a fundamental issue is how to make enough number and quantity of recombinant proteins. There are two major cloning strategies, summarized in Fig. 1, that can create libraries of open reading frames (ORFs) in expression vectors and generate a large number of recombinant proteins in cells (Phizicky et al., 2003). One utilizes a yeast homologous recombination system, in which the PCR products containing yeast recombinant sites are co-transfected into yeast cells with the linearized yeast expression vector (Hudson et al., 1997; Zhu et al., 2000); and the other uses the Gateway recombinational cloning system, which takes advantage of the integration and excision properties of phage λ in *E. coli* (Esposito et al., 2009; Hu et al., 2009). These recombinant proteins are generally tagged with glutathione-S-transferase (GST), Hisx6, tandem affinity purification (TAP), or photocleavable biotin at their N-terminus or C-terminus for ease of purification (Lim and Rothschild, 2008).

Cell-free protein array is another technology that allows protein expression in a large scale on chip without any assistance from cellular functions (Ramachandran et al., 2008). Recently several laboratories have developed many methods, which can basically be characterized into three main categories. (1) Nucleic acid programmable protein array (NAPPA): it uses DNA template that is immobilized onto the same protein capture surface. As shown in Fig. 2, the DNA template is biotinylated and bound to avidin pre-coated onto the protein capture surface. Newly synthesized proteins tagged with GST are immobilized next to the template DNA by binding to the adjacent pre-coated polyclonal anti-GST capture antibody (Ramachandran et al., 2004). Based on this concept, Ramachandran and colleagues developed a high-density self-assembling protein microarray arrayed with up to 1000 unique human proteins (Ramachandran et al., 2008). Also, NAPPA has been used to carry on a comprehensive study of all 262 outer membrane and exported *Pseudomonas aeruginosa* PAO1 proteins (Montor et al., 2009).

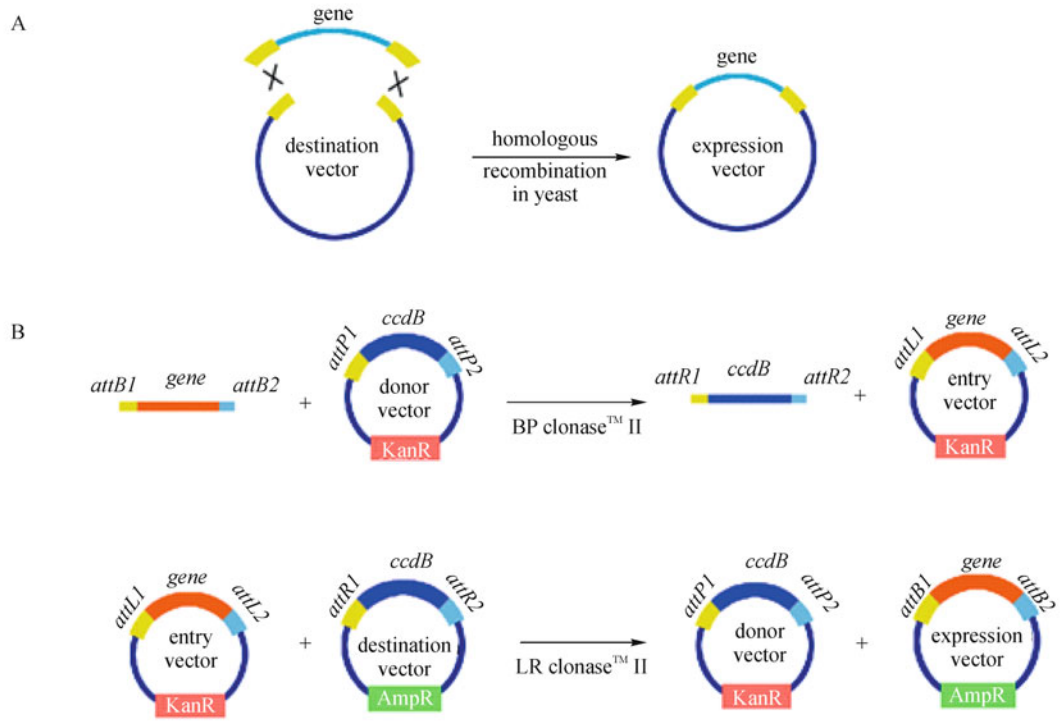

Fig. 1 Two Recombination Systems. A: Yeast Homologous Recombination System: Yeast homologous recombination of a DNA fragment and the linearized destination vector due to the presence of the identical sequences. B: Gateway® Recombination System: The DNA fragment flanked with *att*B sequences (*att*B1 and *att*B2) is combined with a donor vector that contains *att*P1 and *att*P2 sequences and a counter-selectable marker, *ccd*B. Due to the presence of the kanamycin resistant gene in the entry vector, the entry clones are selected on plates containing kanamycin. In a similar fashion, the expression vector is produced by recombination of the entry vector with a specific destination vector that has the *att*R1 and *att*R2 sequences and the same counter-selectable marker *ccd*B. Due to the presence of the ampicillin resistance gene in the expression vector, the expression clones are selected on plates containing ampicillin.

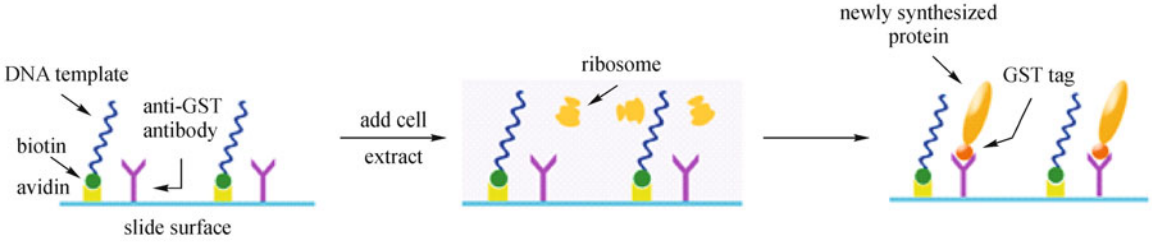

Fig. 2 Schematic diagram of the NAPPA technique. Avidin and anti-GST capture antibody are pre-coated adjacent to each other onto the same protein capture surface. After biotinylated DNA templates are bound to avidin, cell extract is added onto the surface for transcription and translation. The newly synthesized GST fusion proteins are captured by the adjacent anti-GST capture antibody to form the array.

(2) Protein *in situ* array (PISA): multiple spotting technique was developed to allow the generation of high density protein microarrays from unbound DNA template molecules (Angenendt et al., 2006). As depicted in Fig. 3, DNA constructs produced by PCR are deposited on the activated slides in the first spotting run and the cell-free transcription and translation mixture is transferred on the top of the very same spot in the second spotting run. Proteins with/without fusion tags can be produced by this method to fabricate protein microarrays. (3) DNA array to protein array (DAPA): Figure 4 illustrates that the technique starts with the spotting and immobilization of an array of DNA templates onto a glass slide. The slide is then assembled face-to-face with a second slide pre-coated with a protein-capturing reagent, and a membrane soaked with cell extract is placed between the two slides for

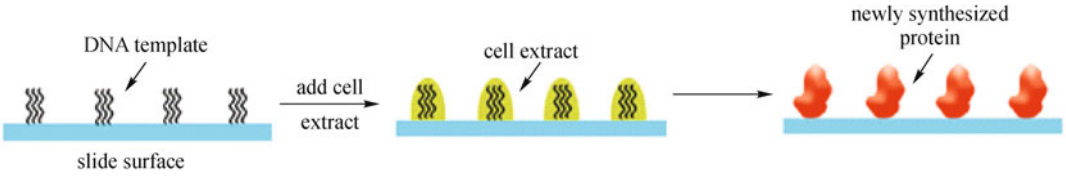

Fig. 3 Schematic diagram of the PISA technique. DNA templates are deposited on the activated slides at the first spotting run, and cell extract for transcription and translation is dotted on the top of each spot at the second spotting run. Subsequently, newly synthesized proteins with/without fusion tags are generated and form the array.

Fig. 4 Schematic diagram of the DAPA technique. DNA templates are spotted onto a glass slide. The slide is assembled face-to-face with a second slide pre-coated with a protein-capturing reagent, and a membrane soaked with cell extract is placed between the two slides for transcription and translation to take place. The newly-synthesized tagged proteins diffuse through the membrane and become rapidly immobilized on the capture slide surface to form the array.

transcription and translation to take place. The newly-synthesized His-tagged proteins diffuse through the membrane and become rapidly immobilized on the capture slide surface to form the array (He et al., 2008).

Protein fabrication on solid support is another challenge to produce protein microarrays. For a protein without a fusion-tag, two immobilization approaches are available, either the protein is passively adsorbed onto the glass surface pre-coated with nitrocellulose, gel pads, or poly-L-lysine, or it is covalently bound to the glass surfaces pre-coated with amines, aldehyde, or epoxy via amines. The approaches immobilize proteins in random orientations. However, if protein configuration in certain orientation is required for scrutinizing the protein interactions, the proteins with fusion-tag are useful for immobilization. For example, nickel coated slides are applied to Hisx6 tagged proteins; Strep tag facilitates the oriented immobilization of recombination proteins onto streptavidin functionalized surfaces; and biotinylated proteins can be immobilized onto avidin-functionalized glass slides.

The captured molecules, such as DNAs, lipids, carbohydrates, or proteins, on protein microarrays can be detected by direct labeling or a secondary labeled antibody. The detection signatures, such as fluorescent, photochemical, or radioisotope tags, are available for labeling. Fluorescent labeling is the most popular method due to its feasibility in operation as well as its sensitivity in detection (Hall et al., 2007). Recently there have been some new detection methods applying physics principle for protein microarray, including surface plasmon resonance (SPR), carbon nanotubes, and carbon nanowires (Chen et al., 2008; Lausted et al., 2009). Although these physics detection technologies are still in their infant stage and unavailable for high-throughput proteomics, they have some incomparable advantages over biochemical or chemical labeling, because no specific reaction or reagent is required for physics approaches (Hall et al., 2007).

3 Applications of forward protein microarrays

3.1 Protein–lipid interactions

The mobile lipid molecules can adhere to the membrane protein surface and flexibly adjust the protein conformational changes as well as structural rearrangements. During protein–lipid interactions, the specific lipid species confer protein stability and folding status. It is believed that protein–lipid interactions are involved in assembly or oligomerization of multisubunit complexes or supercomplexes, which directly affect the functions of membrane proteins. With 5800 yeast proteins, Zhu et al. studied protein–lipid interactions and discovered new calmodulin and phospholipid binding proteins (Zhu et al., 2001); Gong et al. demonstrated that receptor binding modules could

form high affinity interactions with lipid headgroups outside a membrane environment, and found the PIP(n)-binding proteins as well as the affinity of protein-PIP(n) binding interactions (Gong et al., 2009).

3.2 Protein–DNA interactions

Stansfield et al. employed commercially available protein microarrays to screen additional interactions of cisplatin-modified DNA, and identified many novel protein–DNA interactions (Stansfield et al., 2009). A protein microarray containing 4256 proteins encoded from *Escherichia coli* K12 strain was used to develop assays for identifying proteins involved in the recognition of potential base damage in DNA (Chen et al., 2008). By using a group of DNA probes each containing a mismatched base pair or an abasic site, Chen et al. found a small number of proteins that could recognize each type of probes with high affinity and specificity. Ho et al. made a microarray of 282 yeast transcription factors and utilized it to probe the oligonucleotides of evolutionarily conserved sequences (Ho et al., 2006). Combination of bioinformatics and protein microarray data is able to develop a new data set characterizing the human protein–DNA interactome (Hu et al., 2009).

3.3 Protein–RNA interactions

A protein microarray with more than 5000 *Saccharomyces cerevisiae* proteins was taken to screen the proteins that could preferentially bind a small RNA hairpin with clamped adenine motif (CAM). Zhu et al. demonstrated the proteome array to be powerful in identification of the specific RNA-binding proteins for antiviral activities (Zhu et al., 2007).

3.4 Drug–protein interactions

To detect the potential protein targets of drugs, Feyen et al. generated a protein microarray with 384 different human proteins; two therapeutic antibodies (adalimumab, infliximab) and one receptor fusion protein (etanercept) were applied to the chip for screening the drug–protein interactions (Feyen et al., 2008). The preliminary data revealed that a significant number of proteins were recognized by tumor necrosis factor-alpha inhibitors.

3.5 Protein–protein interactions

A microarray containing *Arabidopsis thaliana* proteome was used to identify the proteins interacted with calmodulin (CaMs)/CaM-like (CML). Popescu et al. found a large number of novel CaM/CML targets, including transcription factors, receptors, intracellular protein kinases, F-box proteins, RNA-binding proteins, and proteins of unknown function (Popescu et al., 2007).

3.6 Protein–carbohydrate interactions

To further understand the roles of protein glycosylation in eukaryotes, Kung et al. globally identified glycan-containing proteins in yeast (Kung et al., 2009). A fluorescent lectin binding assay was developed and used to screen protein microarrays containing over 5000 proteins purified from yeast. A total of 534 yeast proteins, including 406 novel ones, were identified to possess the capacity of binding to either Concanavalin A (ConA) or Wheat-Germ Agglutinin (WGA). Of the novel glycoproteins, 45 were validated by mobility shift upon treatment with EndoH and PNGase F (Kung et al., 2009).

3.7 Enzyme–substrate interactions

Utilizing yeast proteome microarray, Tao et al. screened the substrates of poly(ADP-ribose) polymerases (PARPs) that catalyze polyADP-ribosylation of nuclear proteins, and identified 33 putative PARP-1 substrates, 6 of which were involved in ribosome biogenesis (Tao et al., 2009). Lin et al. utilized yeast proteome chip to identify the nonchromatin substrates for the essential nucleosome acetyltransferase of H4 (NuA4) complex (Lin et al., 2009). Phosphorylation usually results in a functional change of the target proteins due to alterations in enzyme configuration, cellular location, or association with other proteins. Protein microarray provides a high-throughput method to identify potential kinase substrates. Zhu et al. generated an Epstein-Barr virus (EBV) protein array to evaluate the targets of the EBV protein kinase BGLF4, and found several BGLF4 substrates involved in EBV lytic DNA replication and virion assembly, which were previously unrecognized (Zhu et al., 2009). Using a protein microarray, Schnack et al. identified a novel substrate of Cdk5/p25, protein phosphatase 1 regulatory subunit 14A (PPP1R14A), which is important for the physiological function of Cdk5 in synaptic signaling (Schnack et al., 2008).

3.8 Autoantibody profiling for autoimmune diseases

An autoantibody is an antibody manufactured by the immune system that is directed against one or more of the individual's own proteins. Many autoimmune diseases, notably lupus erythematosus, are caused by such autoantibodies. Protein microarrays have great potential for identifying autoantibodies, which leads to development of new approaches in diagnosis, prognosis, and medical treatment of such diseases. Chen et al. described the use of a whole *Escherichia coli* proteome microarray as a novel high throughput proteomic approach to screen and identify new serological biomarkers for inflammatory bowel disease (IBD) (Chen et al., 2009). The study pointed out that specific antimicrobial antibodies in the sera of patients with IBD were valuable serological biomarkers for

diagnosis/prognosis of the disease. Song et al. profiled the autoantigen repertoire of patients with autoimmune hepatitis (AIH) *versus* those with other liver diseases on homemade 5000 human protein arrays, and identified three new antigens, RPS20, Alba-like, and dUTPase, as highly AIH-specific biomarkers, with sensitivities of 47.5% (RPS20), 45.5% (Alba-like), and 22.7% (dUTPase) (Song et al., 2010).

Cancer cells in the patient body can present either novel proteins or abnormally large amounts of normal proteins. The immune response to these proteins can also lead to the production of autoantibodies. For instance, Babel et al. examined 20 sera from colorectal cancer (CRC) patients and healthy subjects on commercial protein microarrays containing 8000 human proteins (Babel et al., 2009). Their studies confirmed the presence of specific autoantibodies for CRC and revealed new individual markers of the disease, such as PIM1, MAPKAPK3, and ACVR2B, to be potentially useful in diagnosis of CRC with higher specificity and sensitivity than previously reported serum biomarkers. Patwa et al. used a natural protein array to study the humoral response in the sera from pancreatic cancer patients (Patwa et al., 2009), and clarified that phosphoglycerate kinase-1 and histone H4 could elicit a significant differential humoral response in cancer sera compared with age- and sex-matched sera from normal patients and patients with chronic pancreatitis and diabetes.

3.9 Profiling the antibodies against infection diseases

Understanding the way in which the immune system responds to infection is central to the development of vaccines and many diagnostics. To provide insight into this area, Felgner et al. fabricated a protein microarray containing 1205 *Burkholderia pseudomallei* proteins, and applied the chip to probe 88 melioidosis patients' sera (Felgner et al., 2009). A total of 170 reactive antigens were identified, including 49 antigens to be significantly more reactive in melioidosis patients than in healthy people and patients with other types of bacterial infections (Felgner et al., 2009). Furthermore these biomarkers were referenced to classify melioidosis positive and negative individuals with sensitivity and specificity of 95% and 83%, respectively. Using malaria as a model with the protein chip containing a panel of 250 Plasmodium falciparum (Pf) proteins, Doolan et al. profiled the antibodies that developed after natural or experimental infection or after vaccination with attenuated organisms, and identified 72 highly reactive Pf antigens in the infection sera (Doolan et al., 2008). Li et al. used a protein microarray containing 144 known or putative virulence-related proteins of *Yersinia pestis* to evaluate the antibody responses of plague patients (Li et al., 2008). Antibodies against 14 of the bacterial proteins were detected in all patients, providing potential candidates for novel protective antigens and novel serodiagnostic markers in *Y. pestis*.

3.10 Antibody specificity determination

Antibodies are routinely used as research tools, diagnostic assays, and therapeutic reagents. Ideally, these applications require antibodies with high sensitivity and specificity; however, many commercially available antibodies are limited in their use as they cross-react with non-related proteins. High-density protein array technology is a fast and effective means for determining the specificity of antibodies and can be used to screen for highly specific antibodies for further applications. Hu et al. identified the corresponding antigens for five highly specific mAbs via screening on a protein microarray of 1058 unique human liver proteins (Hu et al., 2007). In Kijanka's study, 6 commercially available monoclonal and polyclonal antibodies were screened on high-density protein arrays comprising of ~10 000 recombinant human proteins (Imagenes) (Kijanka et al., 2009). Two of the 6 antibodies, anti-pICln and anti-GAPDH, bound exclusively to their target antigens and showed no cross-reactivity with non-related proteins. However, 4 of the antibodies, anti-HSP90, anti-HSA, anti-bFGF and anti-Ro52, displayed strong cross-reactivity with other proteins on the array.

4 Applications of reverse protein microarray

Pirnia et al. established a 3-D *ex vivo* culture technique in combination with reverse-phase protein microarrays (RPPM) as a novel tool for cancer research, in which the cancer tissues and the respective corresponding normal tissue controls from patients with CRC were cultured *ex vivo* (Pirnia et al., 2009). At different time points, the cultured samples were processed into lysates and analyzed on RPPM to assess the expression of carcinoembryonic antigen (CEA) and other 24 proteins involved in the regulation of apoptosis. Their results demonstrated that reverse protein microarray is useful for investigating complex patterns of protein expression and modification over time. Another typical case is the Molero's study. To better understand how *Salmonella* invades host enterocytes and how the bacterial proteins contribute to re-programming host cell pathways, the group employed ZeptoMARK reverse-phase protein array technology. They printed 32 sample lysate on arrays and used phospho-specific antibodies to evaluate the phosphorylation of signaling proteins (Molero et al., 2009). This is the first time that reverse-phase protein array technology is used in the cellular microbiology field, demonstrating its value to screen for host signaling events through bacterial infection.

5 Applications of antibody microarray

Antibody microarrays have great potential and significant value in biological research. Particularly, cancer research could benefit from the unique capabilities of this technology. Weber et al. generated a protein microarray by printing antibodies against 83 proteins onto membranes and characterized the protein profiles of head and neck squamous cell carcinomas (HNSCCs) (Weber et al., 2007). Of the 83 proteins examined, 14 were found differentially expressed between HNSCCs and normal epithelium, with 8 up-regulated and 6 down-regulated (Weber et al., 2007). The differential expression of these proteins was confirmed using Western blot and tissue microarrays. Antibody microarray analysis of complex proteomes provides a useful tool to define cancer associated protein signatures. Ingvarsson et al. utilized a recombinant scFv antibody microarray to classify sera derived from pancreatic adenocarcinoma patients versus healthy subjects (Ingvarsson et al., 2008). A protein signature based on 19 nonredundant analytes was discriminated between cancer patients and healthy subjects in this study. Carlsson et al. made a large-scale recombinant scFv antibody microarray in an attempt to classify metastatic breast cancer versus healthy controls, based on differential protein expression profiling of whole serum samples (Carlsson et al., 2008). Using this multiplexed and miniaturized assay, breast cancer could be classified with a specificity and sensitivity of 85% based on 129 serum analytes. When a subgroup of patients, not receiving anti-inflammatory drugs, was analyzed, a novel 8 biomarker signature with a further improved predictive power was indicated. Uemura et al. conducted a proteomics study using an antibody microarray consisting of 725 antibodies and identified 24 proteins with aberrant expression in esophageal cancer compared with the corresponding normal mucosa (Uemura et al., 2009). In a longer perspective, antibody microarray analysis could provide a tool for the development of improved diagnostics and intensified biomarker discovery for cancer patients.

Antibody microarray can also be used to analyze the biomarkers in other diseases. Using antibody microarrays, Lal et al. compared microparticle CD antigen expression in acute coronary syndrome and healthy subjects and found that 10 CD antigens (44, 45, 54, 62E, 79, 102, 117, 130, 138, and 154) had significantly increased expression in the disease group relative to the healthy controls (Lal et al., 2009). These results were then verified using flow cytometry and scanning electron microscopy. Zander et al. applied the Sigma Panorama Antibody Microarray-Cell Signaling Kit to identify differential protein expression in nasal polyps from aspirin-sensitive (AS) *versus* aspirin-tolerant (AT) patients with chronic rhinosinusitis (CRS) and CRS with nasal polyps (CRSwNPs) (Zander et al., 2009), and found a greater than 2-fold change in expression of both beta-adaptin and heat shock protein 70 (HSP70) in AS. Western blot analysis confirmed up-regulation of beta-adaptin and HSP70 in nasal polyp tissue from AS patients.

6 Conclusions

Protein microarray technology offers a powerful tool to elucidate the complexity of the proteome and study protein interactions with other molecules in a large scale. The information elicited from protein microarray will give us an insight to the fantastic complexity of life at molecule level. Screening technology with protein microarray can also provide a fast and overall view for development of drugs, vaccines, and diagnostic/prognostic biomarkers. Although tremendous progress has been made in generation and application of protein microarray, there is still a need for computational methods to analyze large data sets and to integrate complex and disparate kinds of protein information. And it is a big challenge to best convert the broadly proteomic discovery into deeply functional understanding of protein. Doubtlessly, we believe that protein microarray has a great and unique technical advantage, and will become a routine tool for life science after some bottlenecks are overcome in near future.

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