HOW THE INNOSCAN SCANNERS AND MICROARRAYS ARE BEING USED TO COMBAT INFECTIOUS DISEASES

ABSTRACT

Viruses, bacteria and parasites can cause deleterious diseases requiring rapid and efficient detection and therapeutic tools. Emerging infectious diseases with little known mechanisms of infection and pathogenicity represent a big challenge for research and clinics. The discovery of host-pathogen interactions, pathogen epitopes and patient immune responses are essential in order to develop new preventive or therapeutic approaches such as vaccines and new drugs. In a similar way, epidemiological studies handle big patient cohorts for which automation and high throughput are needed. On that note, microarrays and the Innoscan's fluorescent detection can be used at any step of the infectiology research, from pathogen detection to vaccine validation. Using dedicated glycan arrays, researches are now able to define precise mechanisms of virus and bacterial infection. In a similar way, high density peptide arrays are precious tools for understanding immune response, defining immunogenic epitopes, and for designing and validating vaccines

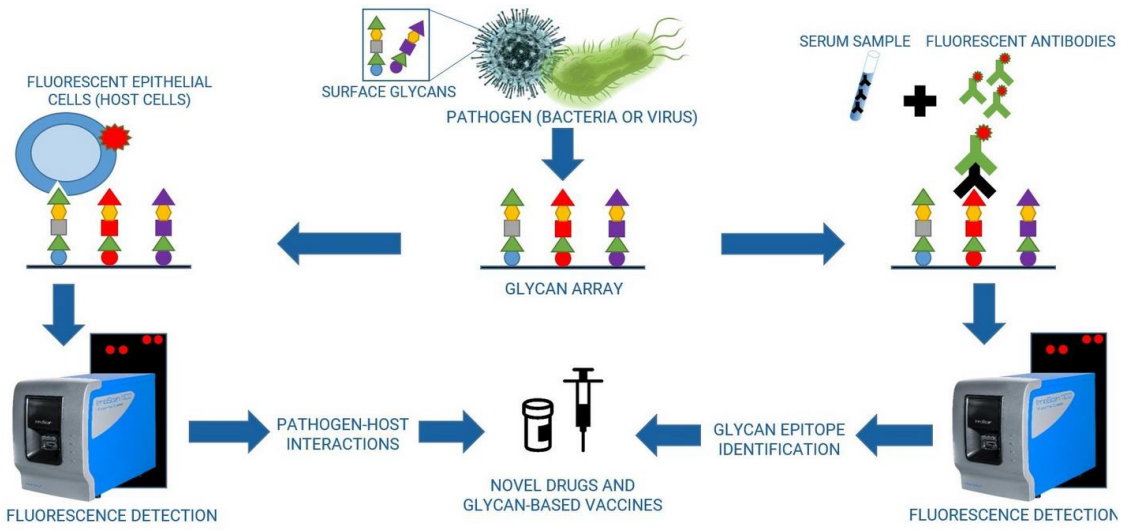

Figure 1. Graphical abstract. Example of glycan microarray use on infectiology

MICROARRAY BASICS

Microarrays are miniaturized tests composed of biomolecules (nucleic acids, protein, peptides, glycans, etc.) precisely arrayed at a known position on a surface, usually a microscope slide. Samples, such as patient sera, cell lysates, or purified DNA are incubated onto the microarray. Then, complementary molecules of the samples specifically interact with immobilized molecules on the microarray. Fluorescence labeling is usually used to follow these interactions. Using fluorescent scanners such as the *InnoScan*, it is possible to detect fluorescence with increased sensitivity and high dynamic range to gather reliable data. Microarrays are superior to other techniques due to their ability to simultaneously detect multiple interactions with high throughput

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capability to test large sample sets, using low volumes of reagents and samples.

INFECTIOUS AGENTS DETECTION AND DISEASE DIAGNOSTICS

DNA microarrays can detect infectious agents such as viruses or bacteria. Multiplexing capabilities of microarrays allow for the detection of different viruses in a single slide. A DNA microarray can test 100 different viral species simultaneously. In 2015, Martinez *et al*[1] designed a DNA microarray for the simultaneous detection of gastrointestinal viruses involved in children gastroenteritis. The authors validated the use of DNA microarray to detect 10 different virus species, with similar or higher sensitivity than RT-PCR. The researchers also pointed out the utility of DNA microarrays for defining coinfection with more than one virus, helping in the understanding of the etiology of gastroenteritis in children. Similarly, the SMAvirusChip developed by Khan MJ *et al* is a DNA microarray designed to detect viruses transmitted by small mammals and arthopods[2]. The SMAvirusChip v2 contains 8 blocks each with 15,000 spots of 60-mer oligonucleotide probes capable of detecting 416 viruses, including Dengue (DENV) and Zika viruses. Authors tested the sensitivity of the SMAvirusChip v2 using serial dilutions of DENV and compared it to classical RT-PCR. They show a one $\log_{10}$ lower sensitivity than real-time RT-PCR with a limit of detection as low as 2.6E3 RNA copies/ml. Together these studies demonstrate how effective using DNA arrays as a surveillance and screening tool can be for the detection of viruses in infected patients.

DNA arrays also provide methods for the surveillance of animal viruses having a deleterious impact on economics. Recently, Sultankulova *et al*. designed an oligonucleotide microarray for the diagnosis of avian viral diseases. They use the *InnoScan 710* scanner to detect fluorescence signals defining the presence of different avian viruses on bird samples[3]. This test was successfully used for a wide study aimed at understanding the molecular epidemiology of avian avulavirus 1 in wild birds in Kazakhstan[4]. The study included 860 cloacal swab samples from 37 bird families in nine Kazakhstan regions. It was possible to define the virus genotype and build a cartography of different virus abundance. This work shows the potential of DNA oligo arrays as high throughput tool for epidemiological studies and virus surveillance.

Bacteria serotyping using either DNA or protein arrays is useful to differentiate between bacteria eliciting different immune responses. In 2010 Marimon *et al.* successfully developed an antibody microarray to perform *Streptococcus pneumoniae* serotyping[5]. This protein serotyping microarray consists of serotype-specific antibodies targeting the 83 or the 91 *S. pneumonia* serotypes. The specific bacteria-antibody binding is then followed by fluorescent antibodies. The *InnoScan 710* scanner and *Mapix* software were used to detect and quantify fluorescence, respectively. Compared to the Quellung technique, the standard method for bacteria serotyping, the antibody microarray shows better specificity and shorter assay time. Furthermore, the multiplexing capabilities of antibody microarrays allows for the detection of cross-reacting antibodies in the same assay, which is one of the limitations of classical methodologies.

Several diseases are difficult to diagnose due to the absence of specific physical symptoms or clinical markers. For example, upper respiratory disease can be caused by a wide variety of viruses giving the same clinical symptoms. The diagnostic difficulties can complicate patient monitoring and impact the epidemiological surveillance. To address this issue, Marina Plotnikova form the Smorodintsev Research Institute of Influenza at St. Petersburg, Russia, developed an antibody microarray capable of differentiating between six common viruses causing upper respiratory disease: IAV, IBV, RSV, hAdV, hPIV2, and hPIV3[6]. The assay, which is a miniaturized multiplexed ELISA-like sandwich technique, shows limits of detection slightly lower than those observed with classical ELISA. The potential of their assay is reliant on multiplexing and time-saving assays. Authors claimed the possibility of expanding the number of pathogens simultaneously detected to about 50.

HOST-PATHOGEN INTERACTION STUDIES

In order to develop adapted strategies against pathogen infection, it is necessary to

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understand the mechanisms by which pathogens cause infection. Virus infection is generally initiated by one or several specific interactions between viral proteins (capsid proteins or glycoproteins) and surface host cell receptors. While bacteria pathogenesis is mediated by bacteria surface glycoproteins, which sometimes enable them to evade human immune system. Glycans are therefore essential in defining the virulence of pathogenesis, through the interactions between virus or bacteria glycan binding proteins (GBP) with host cell membrane glycans. Glycan arrays give insights into these interactions, providing leads in drug and vaccine developments.

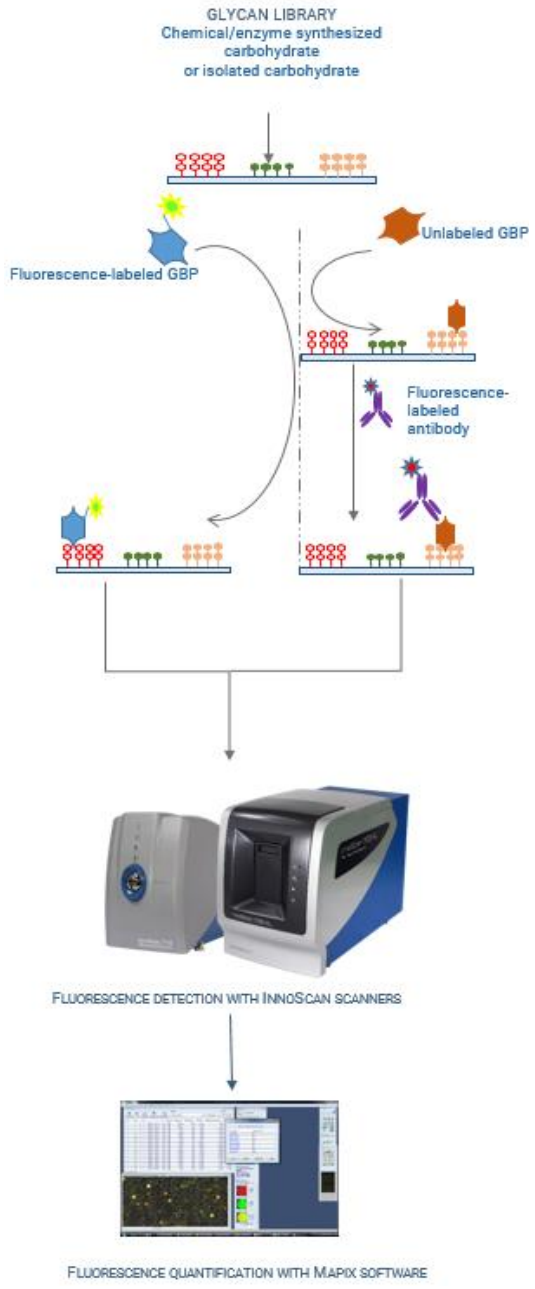

Figure 2. Glycan array workflow

A typical glycan array workflow begins with the glycan array construction. Glycans or lectins are immobilized on functionalized glass slides using a microarray spotter. The immobilization occurs by either covalent binding or adsorption depending on the printed substrate. This glycan array serves to catch glycan-binding molecules (either GBPs, virus, cells, or others) from a sample. Fluorescence dyes allows for the detection of the interaction between GBPs and the immobilized glycans. When the GBP is coupled to a fluorophore before the incubation step, or once the incubation step is done, a fluorescently-labeled antibody against the GBP is used in an additional step. Fluorescence is then detected using a microarray scanner. The Innopsys InnoScan is the ideal tool for automating fluorescence detection with the ability to detect either two or three dyes simultaneously, while Mapix software analyzes images to quantify fluorescence in a complete automated process (Figure 2).

Glycan arrays are used to characterize and monitor the influenza virus. McBride et al from the Scripps Research Institute developed a 48-array slide to monitor the Influenza A virus hemagglutinin avidity specificity[7]. This microarray simultaneously detects the interaction of 6 different glycans with 6 GBPs at 8 dilutions. The assay aims to monitor whether avian viruses are adapting to human-type receptors. Authors used the InnoScan 1100 for their assays. They use the simultaneous detection of three channels to follow three different kinds of spots on their slides: They use Atto488-NHS dye during the slide manufacturing process as grid marker/landing lights, streptavidin-555 dye to look at biotinylated lectins with known specificity as controls; and Alexa 647-labeled antibody to detect receptor-binding specificity of influenza A virus hemagglutinins. The resulting assay gives results equivalent to standard-plate assays, but with a significant decrease in consumption of compounds and biologicals by 1,500x and 360x, respectively (Figure3).

As for viruses, bacterial lectins modulate the adherence to host tissue mediating

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colonization and infection. The study of bacteria-host tissue interactions is then essential to understand and contrive bacterial infection. Since glycans are complex

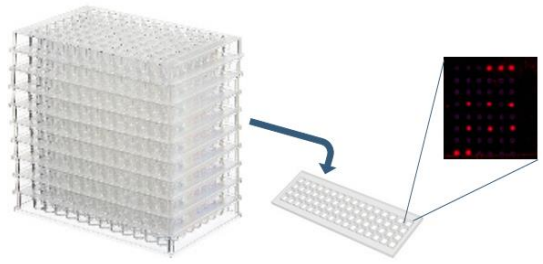

Figure 3: It was evaluated that the use of a 48-well microarray for the simultaneous evaluation of six glycan binding proteins at 8 dilutions was equivalent to 18 96-well plates of a traditional avidity assay. The biochip would reduce reagent consumption by 360 (McBride et al [7]).

molecules, methodologies addressing the vast diversity and complexity of glycan-lectin interactions are needed.

Glycan arrays using intact cells are effectively tools for defining several bacterial lectin interactions with host glycan structures. Using a fluorescence-labeled intact bacteria instead of purified proteins, researchers are able to follow the interaction of bacterial lectins with host-candidate glycan structures. De Olivera *et al.*[8] revealed insights on differences between *Streptococcus pyogenes* infection rates related to host glycosylated structure diversity using whole cell glycan arrays. They found that M1 bacterial protein showed high affinity towards several terminal galactose blood group antigen structures. Differences in blood group antigen expression were associated with different bacteria adherence to host-cells, explaining the link between host blood group antigen expression and M1T1 Group A streptococcal (GAS) colonization. Recently, researchers from the Wollongong and Queensland Universities, in collaboration with the Institute of Glycomics from the Griffith University in Australia, published the full methodology to create glycan arrays and investigate the interactions of GAS with host glycan structures[9]. They use the *InnoScan 1100* to detect either fluorescent-labeled purified proteins or intact fluorescent-labeled bacteria. Glycan arrays using intact bacteria give a more comprehensive analysis of bacterial adherence to and colonization of host organs.

SCREENING HOST IMMUNE RESPONSE TO INFECTION

During infection, pathogen-host interactions trigger host immune response aimed at controlling pathogen colonization and disease. Innate immune response include the production of pro-inflammatory molecules such as cytokines and chemokines. This non-specific reaction to infectious agents is a rapid and, in general, effective process to contain infection. When infectious agents succeed in evading the innate immune response, specialized immune cells such as T-cells and B-cells are activated, hence initiating the adaptive immune response.

Inflammation is then one of the first mechanisms against infectious agents. However, exacerbated and uncontrolled inflammation causes host tissue damage. Cytokine and chemokine secretion profiling helps in the understanding of innate immune response mechanisms and in getting insights on how to control inflammation and avoid tissue damage. On this scope, researchers from Shijiazhuang Aier Eye Hospital in China used antibody microarrays and the *InnoScan 300-G* (now *InnoScan 710-G*) to study the cytokine secreted by human keratocytes during the herpes simplex virus type 1 (HSV-1) infection[10]. They were able to stablish that the secretion of 18 inflammation-related cytokines and chemokines was upregulated in HSV-1-stimulated keratocytes compared with non-infected keratocytes. Furthermore, they found that the specific inhibition of p38 MAP kinase, diminished the production of 10 pro-inflammatory cytokines. These findings give therapeutic insights to modulate HSV-1 infection and avoid excessive scarring and tissue damage caused by immoderate innate immune response.

ANTIBODY PROFILING

Antigen pathogen presentation is the starting point of adaptive immune response to infection. An antigen is defined as a substance that generates a specific immune response leading to the generation of a specific antibody against it. Different antibody isotypes operate in distinct

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places and have different roles. During infection, IgM are the first antibodies to be produced in a humoral immune response. It has lower affinity than other immunoglobulins. Its rapid production helps quicken control over infections by activating the complement system. IgG and IgA have higher affinity towards the individual antigen-binding sites. IgG is the principal isotype in the blood and extracellular fluid, whereas IgA is the principal isotype in secretions. Antibody subpopulation profiling may give insights into adaptive immune responses to infection, identify target antigens and define specific treatments.

Protein, peptide or glycan microarrays can help in profiling antibody isotype responses. While the majority of experiments focalize on proteins and peptides, the use of glycan arrays could also lead to a deeper and more reliable knowledge of adaptive immune response. In fact, carbohydrate-binding antibodies are involved in many immunological processes and have been shown to be useful in clinical diagnosis.

Glycan microarrays are therefore powerful tools to test and compare different isotypes of anti-carbohydrate antibodies. In 2018, Sara Durbin from Prof. Gildersleeve group at the National Cancer Institute in Maryland, developed a multiplexed glycan array to test and compare human serum anti-glycan IgA, IgG and IgM repertories on a single experiment[11]. They took advantage of the three-color detection of the *InnoScan 1100* to detect the three antibody isotypes simultaneously. The glycan array consists of a spotted microarray with 500 glycans and glycoproteins, including controls. They used the 480 nm channel to detect IgA, the 532 nm to measure IgG and the 635 nm laser to detect IgM. They developed an ingenious methodology to overpass some fluorophore signal bleeding between channels in order to do a direct comparison between antibody isotype abundance and glycan-specificity.

EPITOPE MAPPING AND VACCINE DEVELOPMENT

While antigens are complex molecules, usually proteins or glycoproteins, only some parts of the molecule bind to antigen-specific membrane receptors of lymphocytes or to antibodies. These immune immunological active regions of the antigen are called

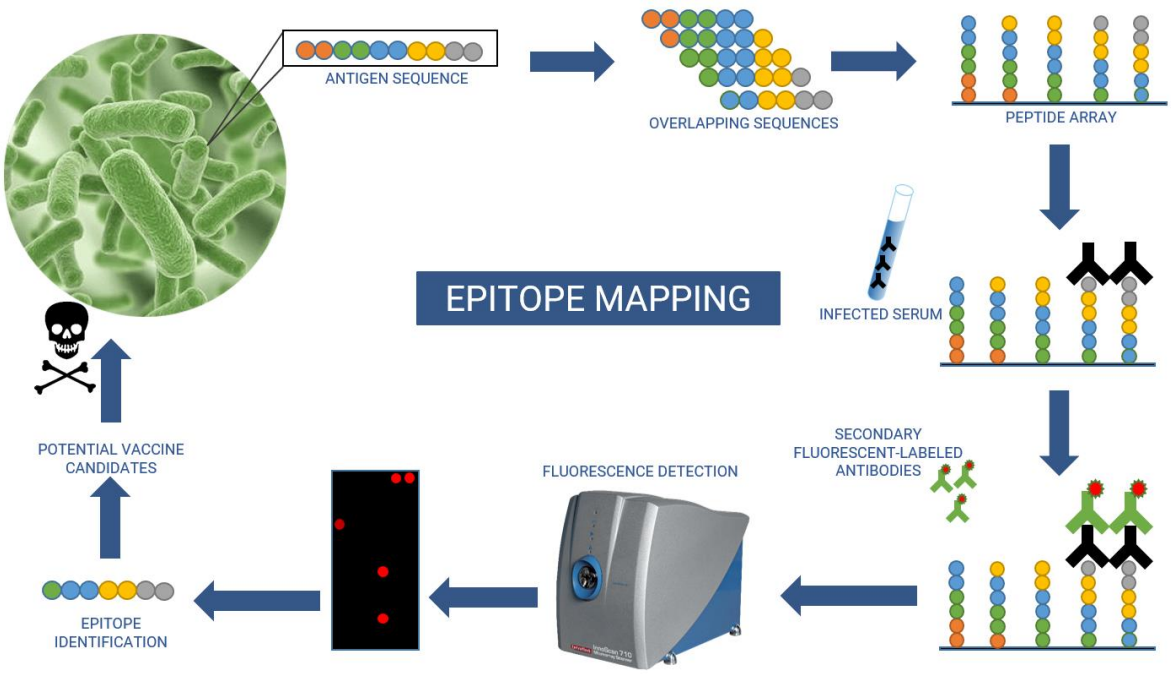

Figure 4. Epitope mapping application to vaccine development

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epitopes, which are usually peptides or glycopeptides.

The identification of epitopes and epitope-antibody interactions is thus important to understand the adaptive immune response and to further design effective synthetic vaccines and diagnostics tools. The Medical Subject Headings (MeSH) defines epitope mapping as the different methods used for studying the interactions between antibodies with specific protein epitopes[12].

Among the different methods for epitope screening, peptide arrays present significant advantages as efficient and high-quality analyses. Working with peptide arrays enables one to overcome the challenges of protein synthesis and purification while providing high-throughput analysis of peptide interactions. Peptide arrays are composed of peptides spotted on a support, so that each spot of the surface corresponds to one specific sequence. If proteins such as antibodies from samples bind to a spotted one, detection methods can determine which spotted peptide is involved in the interaction (Figure 4). As peptide arrays advance, serum antibodies will be able to bind to thousands or millions of spotted peptides in a single experiment. The interest is to increase the number of probes and test samples with smaller volumes for each screening, while reducing the assay dimensions and costs.

In 2015, Carmona *et al* demonstrated the utility of high-density peptide arrays for the characterization of antibody specificities associated with natural infections[13]. Their HD peptide array consisted of more than 175,000 overlapping 15mer peptides derived from *Trypanosoma cruzi* proteins. Using this HD array and the *InnoScan 900* (now *InnoScan 910*) scanner they were able to discover *T. cruzi*-specific linear B-cell epitopes from clinical samples, showing the potential of peptide arrays as a high throughput tool to study immune response against pathogens.

T. cruzi is the cause agent of Chagas disease, a major health problem in the Americas for which diagnosis is still challenging. One of the immunodominant antigens of *T. cruzi* is a glycoprotein displayed on the surface of infective trypomastigote forms, the trypomastigote small surface antigen (TSSA). The TSSA gene has been show to disclose sequence variations among parasite strains. This interstrain polymorphism has a major impact on TSSA immunogenicity and antigenicity. Using HD peptide arrays, Balouz *et al*. mapped the antigenic structure of TSSA. They were able to define the presence of different partially overlapping B-cell epitopes clustering in the central portion of TSS-CL. From this screening, they defined a 21-amino-acid-long peptide that spans the TSSA-CL major antigenic determinants that were afterwards tested as serological tool for the diagnostics of Chagas disease[14].

The identification of novel epitopes and the understanding of epitope-antibody interaction may give insights into the development of synthetic vaccines. Synthetic vaccines are based on the use of epitopes known to trigger an immune response against a specific virus or pathogen. By introducing a synthetic peptide encompassing the precise regions of the pathogen's epitopes, there would be less unwanted side effects, and less risk of contamination or mutation. According to the World Health Organization (WHO), synthetic peptide vaccines could be a possible alternative

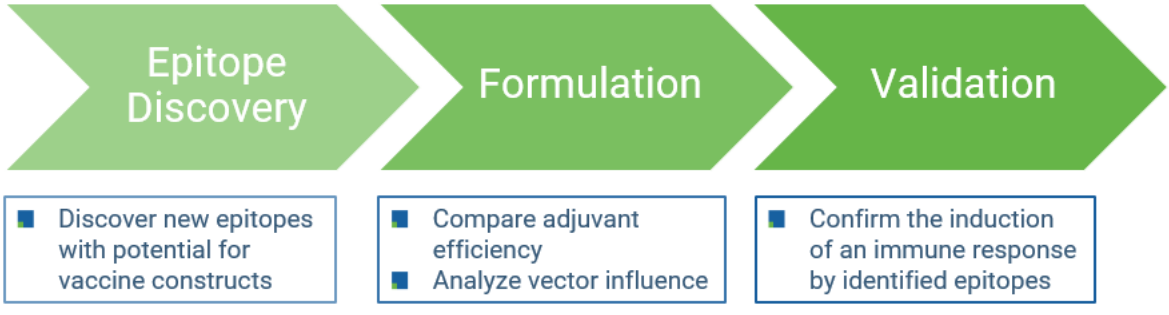

Figure 5. Synthetic vaccine development steps

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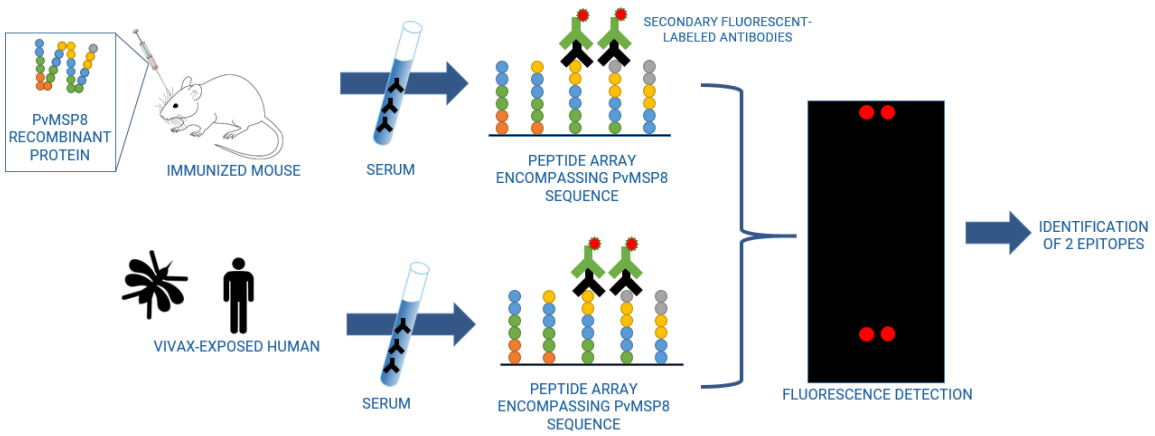

Figure 6. Schematized methodology used by Cheng et al to study the immune respons to Plasmodium vivax merozite surface protein 8 (PvMSP8)[16]

for current vaccination strategies[15]. Vaccine development can be divided into three main steps, as depicted on figure 5: the identification of potential epitopes, the vaccine formulation analysis and the validation of identified epitopes as vaccine candidates.

Epitope mapping is a powerful tool used in identifying epitopes of interest for vaccine development. *Cheng et al* in 2017 studied the immune response to *Plasmodium vivax* merozoite surface protein 8 (PvMSP8) of naturally-infected patients and in vaccinated mice[16]. To do so, they expressed and purified a recombinant PvMSP8 protein (rPvMSP8) and used to create a linear peptide array in order to map its dominant epitopes. Immune mouse sera, pre-immunized mouse sera, vivax-unexposed human and vivax-exposed human sera's peptide arrays encompassing the PvMSP8 protein sequence were analyzed. Secondary antibodies were used for fluorescence detection with the *InnoScan 300-G* scanner (now *InnScan 710-G*), as schematized on figure 6. Antibodies from vivax-infected patients and rPvMSP8-immunized mouse sera highly reacted with two peptides located at the center of PvMSP8 sequence. Immunogenic assays indicated that rPvMSP8 induces humoral and cellular immune responses against *P.vivax* infection in mouse and human models. Thus, PvMSP8 has immunogenic potential for vaccine development. Similarly, in 2019, *Han et al* identified effective B-cell epitopes of PvMSP1P-19 domain. This domain is present in the *Plasmodium vivax* merozoite surface protein 1 paralog (PvMSP1P). PvMSP1P has a key role in the contact between the parasite antigen and host cells. The peptide array analysis determined two epitopes. These epitopes elicited an immune response with antibodies disrupting the interactions between PvMSP1P and erythrocytes[17]. Thus, either PvMSP8 or PvMSP1P have the potential to be used for vaccine design strategies against plasmodium infection.

Synthetic vaccine development not only depends on the epitope sequence, but also on the ways to carry it and the role of adjuvants. In order to enhance immunogenicity, either virus-carriers or adjuvants are used. It is important to evaluate the impact of virus carriers and adjuvants on the specificity and the quality of a vaccine-induced antibody response. Linear peptide arrays are then used to test the impact on the specificity of a vaccine due to different vaccine formulations. On this scope, the group headed by Kristina De Paris from the University of North Carolina at Chapel Hill performed two different studies to define the impact of viral-vectors[18] and of novel adjuvants[19] on the immunogenicity of HIV vaccine on infant rhesus macaques. They used linear peptide arrays with fluorescence-labelled secondary antibodies to detect antibody-epitope interaction specificity and magnitude. To define the binding magnitude, they scanned using the *extended dynamic range (XDR)* mode provided by the *InnoScan*. Then the fluorescence values for pre-immune samples were used as blanks and subtracted from the post-immunization values for each peptide. The binding magnitude was

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then defined as the highest binding to a single peptide within the epitope region.

by different vaccine adjuvants[19]. The array contained peptides encompassing the HIV gp120 clade sequence. While authors were able

Table 1. InnoScan use on infectiology research

APPLICATION	MICROARRAY TYPE	SCANNER MODEL	REFERENCES
PATHOGEN IDENTIFICATION	DNA array	InnoScan 710	1, 2, 3, 4
PATHOGEN SEROTYPING	Protein array	InnoScan 710	5, 6
HOST-PATHOGEN INTERACTION STUDIES	Glycan array	InnoScan 1100	7, 8, 9
INFLAMMATION BIOMARKER SCREENING	Cytokine array	InnoScan 710-G	10
ANTIBODY SUBPOPULATION PROFILING	Glycan array	InnoScan 1100	11
EPITOPE MAPPING	Peptide arrays	InnoScan 910 InnoScan 710-G	13, 14 16, 17
VACCINE DEVELOPMENT AND VALIDATION	Peptide arrays	InnoScan 710 InnoScan 1100	18, 19 20

In their first approach Phillips *et al* tested the impact of poxovirus vector on the specificity and the magnitude of HIV gp120 vaccine-induced antibody production on an infant rhesus macaque model[18]. Authors compared the serum samples of four groups: i. before immunization; ii. HIV envelope protein-immunized; iii. Immunized with Poxivirus vector MVA-HIV; and iv. Co-administered with HIV envelope and MVA-HIV. While all the immunized groups showed antibodies recognizing epitopes from other virus clades mainly targeting clade C V3 epitopes; some differences on the proportion of antibodies against other epitope regions between the HIV envelope protein-immunized and the co-administered group were observed. In a similar way, authors were able to define differences in the antibody-peptide specificity when comparing the accelerated and extended vaccination protocols.

In a second study, the same group tested the epitope specificity of the antibodies produced

to detect differences on IgG response depending on the adjuvant-differing vaccine formulation, the epitope specificity saw little to no impact from the different adjuvants. Together, these publications show linear peptide arrays scanned by the *InnoScan* are useful tools for defining the impact of vaccine formulation on its immunogenicity.

Final steps on vaccine development include the validation of the vaccine-elicited response specificity and protective capacity on clinical trials. Since several vaccines are developed on animal models, it is important also to validate the animal model to use when developing a vaccine. Recently, Cheng *et al* compared the epitope specificity of hepatitis C virus-vaccinated humans against immunized-rhesus macaques and mice[20]. They used home-made linear peptide array to validate target epitopes. Their linear peptide array consisted on a library of overlapping peptides covering the HCV-1 E1E2 sequence. A similar set of immune epitopes were recognized by vaccinated

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humans and immunized rhesus macaques, while mice differed, showing the importance of the animal model on vaccine development and the use of linear peptide arrays to validate synthetic vaccine specificity.

CONCLUSION

The present review underlines the use of microarrays and of the *InnoScan* scanners on different areas of infectiology research. Microarrays can be applied to nearly every process from pathogen screening to vaccine development (Table 1). This review show how *InnoScan* users are using microarrays for analyzing infectious agents. DNA microarrays are effective tools in detecting and genotyping infectious agents in patient sera. Whereas protein-, peptide- and glycan-arrays have powerful applications in disease-diagnosis, understanding the mechanisms of infection and screening the immune responses against pathogens.

Fluorescence detection using the *InnoScan* increases the potential of microarray use. The multiplexing capabilities of three-channel simultaneous detection increases the high-throughput capabilities of microarrays. Furthermore, the possibility to perform scans in an *extended dynamic range* allows for the quantitative comparison between a greater variety of samples. Finally, the ease-to-use and robustness of the *InnoScan* scanners makes them most suitable for automatic assays. All of these performance advantages drastically increase the potential of microarrays in medical research, specifically towards fighting infectious diseases.

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