Abstract

The methods for detecting and measuring autoantibodies have evolved markedly in recent years, encompassing three generations of analytical technologies. Many different immunoassay methods have been developed and used for research and laboratory practice purposes, from the early conventional (or monoplex) analytical methods able to detect single autoantibodies to the more recent multiplex platforms that can quantify tens of molecules. Although it has been in use for over 50 years, indirect immunofluorescence remains the standard method for research on many types of autoantibodies, due to its characteristics of diagnostic sensitivity and also to recent technological innovations which permit it a greater level of automation and standardization. The recent multiplex immunometric methods, with varying levels of automation, present characteristics of higher diagnostic accuracy, but are not yet widely diffused in autoimmunology laboratories due to the limited number of autoantibodies that are detectable, and due to the high cost of reagents and systems. Technological advancement in autoimmunology continues to evolve rapidly, and in the coming years new proteomic techniques will be able to radically change the approach to diagnostics and possibly also clinical treatment of autoimmune diseases. The scope of this review is to update the state of the art of technologies and methods for the measurement of autoantibodies, with special reference to innovations in indirect immunofluorescence and in multiple proteomic methods.

Keywords: autoimmune diseases; automation; indirect immunofluorescence; monoplex and multiplex immunoassay methods; standardization.
LE test – reaction of opsonization of anti-nucleosome antibodies) was discovered by means of the common techniques of light microscopy [2]. Next, following the first applications of the method for identifying bacteria using conjugated antibodies with fluorescent tracers, for the first time antinuclear antibodies (ANA) were detected in sera using the indirect immunofluorescence technique (IIF) on antigenic microspots coated to slides [3] and later on cellular substrates made from human tissues and from isolated cells [4].

Thus, IIF was the first monoplex technique applied to the detection of autoantibodies. In some applications however, (i.e., for ANA) it is effectively a multiplex method, given that it is able to detect more than 60 autoantibodies simultaneously, by means of the identification of at least 26 different cellular patterns [5].

Next to IIF, also belonging to the first generation, are other qualitative immunochemical methods (immunodiffusion, complement fixation, passive agglutination, counterimmunoelectrophoresis, immunoprecipitation) developed during the year 1957 (the “golden year of immuno- diagnostics”) [6–11]. During the 1970s and 1980s additional evolution took place in qualitative methods, with the introduction of immunoblot or dot-blot, and second-generation quantitative immunometric assays (IMA) were introduced [radioimmunoassay and its variants, immunoenzymatic assay and its variants, fluoroimmunologic assay, and immunochemiluminescent assay, reviewed in [1]].

Since the turn of the millennium, research has produced an additional rapid advance in diagnostic technology for detection and quantification of autoantibodies. The reasons for this technological revolution have been given as:

(a) the increment of awareness of the physiopatho-
genetic and diagnostic role of autoantibodies in systemic and organ-specific autoimmune diseases (autoantibodies as diagnostic criteria);
(b) the refinement of procedures for identifying and purifying the target autoantigens of the autoimmune reaction, with special reference to recombinant DNA technologies;
(c) the application of quantitative determination of autoantibodies by automated analytic systems, initially introduced for determining hormones, tumor markers, metabolites, antibodies, etc.;
(d) the development of proteomic technologies, able to detect simultaneously a high number of autoantibodies in the same sample (multiplexing).

The occurrence of these processes in clinical laboratories has led to the advent of a new era in the measurement of autoantibodies, with exponential increase in analytic capacity and corresponding rise in the volume of test requests for the diagnosis of AIDs. This evolution has brought about the rapid disposal of 1st and 2nd generation immunoassay methods to the benefit of automated 3rd generation immunoassay methods [12] (Figure 1).

The aim of this review is to define the current state of technologies available in the autoimmunology laboratory (with special concern to the morphologic and proteomic technologies) with a view to updating our previous review [1].

### Multiplex immunoassay platforms: a real improvement of the diagnostic power of autoantibody testing?

From the beginning of the third millennium, based on progress in the development of the genome, multiple measurement of genetic products has become a characteristic of laboratory methodology, defined with the suffix “omics”. Similar to other -omics, (e.g., ribonomics, metabolomics, etc.) proteomics – meaning the science which studies, on a large scale, the expression, the function and the interaction of protein – permits the parallel analysis of hundreds

<table>
<thead>
<tr>
<th>Methods</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st generation monoplex</td>
<td></td>
</tr>
<tr>
<td>Double immunodiffusion</td>
<td>ID</td>
</tr>
<tr>
<td>Complement fixation</td>
<td>CF</td>
</tr>
<tr>
<td>Indirect immunofluorescence</td>
<td>IIF</td>
</tr>
<tr>
<td>Passive agglutination</td>
<td></td>
</tr>
<tr>
<td>Radio-immunoprecipitation</td>
<td></td>
</tr>
<tr>
<td>Western blot</td>
<td></td>
</tr>
<tr>
<td>2nd–3rd generation monoplex</td>
<td></td>
</tr>
<tr>
<td>Radioimmunoassay-Immunoradiometric assay</td>
<td>RIA-IRMA</td>
</tr>
<tr>
<td>Radio-receptor assay</td>
<td>RRA</td>
</tr>
<tr>
<td>Immunoenzymatic assay-Immunoenzymometric assay</td>
<td>ELISA-IEMA</td>
</tr>
<tr>
<td>Immunoblot</td>
<td>IB</td>
</tr>
<tr>
<td>Immunodot</td>
<td>DB</td>
</tr>
<tr>
<td>Chemiluminescence immunoassay</td>
<td>CLIA-ILMA</td>
</tr>
<tr>
<td>Fluorimetric immunoassay</td>
<td>FIA-IFMA</td>
</tr>
<tr>
<td>Multiplex</td>
<td></td>
</tr>
<tr>
<td>Addressable microbeads immunoassay</td>
<td>MBA</td>
</tr>
</tbody>
</table>

Table 1 Classification of immunoassay methods for the detection and measurement of autoantibodies.
of different products, in this case antigens/antibodies, in minimal quantities of biological fluids. In this sense the discipline is held to represent the keystone for thoroughly investigating some acquired human diseases, in the fields of oncology, infectious diseases, immunology, and so on. Consequently, this has assisted the production of numerous analytic methods of both research and commercial application, for the simultaneous quantification of analytes of proteic nature (e.g., hormones, bio-humoral indicators, cytokines, antibodies, autoantibodies, etc.) by using new technologies, some of which can be considered as comprising a new category of immunometric methods [13].

Given the number and complexity of molecules involved in the activities of the immune system, the study of such molecules seemed an ideal field of application for proteomic techniques [14–16], the objective of which is to be able to study the entire autoimmune process rather than their single components. Among the many systems designed, some microarrays have found application for research on autoantibody profiling of AIDs and, in particular, planar and non-planar autoantigenic arrays (in suspension) [1, 17, 18].

Among the planar arrays we note systems made up of microspots on glass slides, on polystyrene microplates or nitrocellulose membranes and linear immunoblot systems on nitrocellulose membranes (Table 2). The first are based on techniques originally proposed by Ekins and Chu [19] and still hold a relevant importance for their simplicity and flexibility. There are not yet commercial applications available for these, but there are notable studies confirming their reliability [20–22].

The second are based on the linear Western blot technique (line immunoblot), with electrophoretic migration on nitrocellulose membranes. The method has found numer-

<table>
<thead>
<tr>
<th>Support</th>
<th>Format</th>
<th>Detection system</th>
<th>Autoantibodies tested (no. and type)</th>
<th>Estimated throughput (test/h)</th>
<th>Commercial product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass slide</td>
<td>Microspot</td>
<td>L</td>
<td>115</td>
<td>~10,000</td>
<td>–</td>
</tr>
<tr>
<td>Glass slide</td>
<td>Microspot</td>
<td>F</td>
<td>196</td>
<td>~10,000</td>
<td>–</td>
</tr>
<tr>
<td>Glass slide</td>
<td>Microspot</td>
<td>F</td>
<td>630</td>
<td>~10,000</td>
<td>–</td>
</tr>
<tr>
<td>Glass slide</td>
<td>Microspot</td>
<td>F</td>
<td>225</td>
<td>~10,000</td>
<td>–</td>
</tr>
<tr>
<td>Microplate</td>
<td>Microspot</td>
<td>C</td>
<td>15</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Microspot</td>
<td>C</td>
<td>30</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Line-blot</td>
<td>C</td>
<td>15 (ENA)</td>
<td>~50</td>
<td>Inno-LIA</td>
</tr>
<tr>
<td>Nitrocellulose membranes</td>
<td>Line-blot</td>
<td>C</td>
<td>13 (ENA)</td>
<td>~50</td>
<td>RecomLine</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Line-blot</td>
<td>C</td>
<td>11 (ENA)</td>
<td>~50</td>
<td>ANA-LIA</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Nanodot</td>
<td>L</td>
<td>10 (ENA)</td>
<td>ND</td>
<td>NALIA</td>
</tr>
</tbody>
</table>

Table 2 Planar autoantigen arrays for autoantibody detection.
C, colorimetry; F, fluorescence; L, chemiluminescence; ND, not declared.
ous commercial applications and there is clinical validation of the systems conducted on large sample numbers, including multicentric studies [23–27]. In general, line immunoblot offers diagnostic and clinical accuracy comparable to, if not superior to, conventional immunometric methods. Nevertheless, it is our opinion that such technologies are not to be considered true microarrays, given the macroscopic dimensions of the transferred proteins and the limits to the number and variety of antigens applicable on the planar solid phase of these systems.

Among the non-planar arrays (Table 3) there have been developed systems in suspension that use microparticles recognized by laser nephelometry [28] or laser fluorimetry in flow cytometry [29]. This last technology offers many commercial applications and has been validated widely by numerous clinical studies, conducted around the world. The principal commercial systems, based on fluorescent microbeads technology, are represented by the following: FIDIS (BioMedical Diagnostics, Marne la Vallee, France); AtheNA Multi-Lyte (Zeus Diagnostics, Raritan, NJ, USA); QuantaPlex (Instrumentation Laboratory, Barcelona, Spain); and BioPlex 2200 (Bio-Rad Laboratories, Hercules, CA, USA) (Table 3).

The FIDIS system, which has a reasonable level of automation and was the first to be introduced on the market, is able to determine diverse autoantibody profiles for connective tissue diseases, antiphospholipid syndrome, vasculitides, rheumatoid arthritis, celiac disease, autoimmune gastritis and autoimmune thyroidities. Many validation studies on its characteristics are published in the literature [30–37].

The AtheNA Multi-lyte system is able to do autoantibody profiling for rheumatic diseases, vasculitides, autoimmune thyroditis and celiac disease. The automation level is similar to the FIDIS method. The many validation studies published attest to its good analytic reliability [36–48].

The QuantaPlex system is able to determine various autoantibody profiles for rheumatic diseases, celiac disease, vasculitides and autoimmune liver diseases and demonstrates, like the first two, high analytic reliability [35, 49–52].

The BioPlex 2200 system is of recent introduction, totally automated and with high analytic productivity, which is able to execute an autoantibody profile for rheumatic diseases, antiphospholipid syndrome, vasculitides and, more recently, celiac disease. Numerous clinical validation studies have been conducted on extensive case populations [53–65].

Enthusiastically welcomed at the time of its appearance in the clinical laboratory a few years ago, multiplexing should have allowed for overcoming several limitations of the conventional methods, including analytic problems (e.g., reduction of the volume of samples and reagents and containment of costs), logistical/managerial problems (e.g., simultaneous measuring in the same session and in the same analyte reaction environment, measured in real time with several methods), and physiopathological problems (e.g., association of markers in pathology-oriented or organ-oriented profiling) [16–18] and should have found extensive application in the routine activities of the autoimmunology laboratory. In any case, at this time the biomedical industry seems oriented to repropose in multiplex versions the autoantibody profiles already consolidated for the principal AIDs, with the only intention being to achieve diagnostic usage limited to the most common autoantibody specificities. This is the principal motive for the slow and extended penetration of these systems into routine diagnostics, where they are substituting for conventional methods. However, a truly flexible autoantibody profile is not yet available to cover the 30–50 most common and important autoantibodies. Such a profile would be used in the laboratory simply for evaluating the autoimmune status and autoantibody profile of the patient, for predictive, monitoring, and therapeutic purposes [66].

The renaissance of IIF

Thanks to the use of various tissue and cellular substrates (e.g., human laryngeal HEp-2 carcinoma cells, *Crithidia luciliae* hemoflagellates, human neutrophil granulocytes, triple-tissue substrate composed of liver, kidney,
and stomach of rat or mouse, monkey esophagus, human umbilical cord, human or animal pancreas, human or animal adrenal gland, pituitary, esophagus, bladder, monkey nerve tissue, etc.), IIF has permitted the detection over the years of an extensive series of specific autoantibodies directed against cellular autoantigens. This method, in time, became a consolidated and universally diffused procedure for detecting patients affected by AIDs, with differentiated use in the different AIDs, according to analytical sensitivity and specificity of the different types of substrates. Currently, the IIF method is considered the reference method for ANA and anti-neutrophil cytoplasmic antibodies (ANCA) screening and a confirmatory test for anti-DNA antibody detection [67 – 70]. However, the method is burdened by some unfavorable features: the need for expert morphologists, the subjectivity of interpretation, and the low degree of standardization and automation [71 , 72]. Because of these limitations and the progressive increase of ANA test requests in autoimmunology laboratories, in the last 15 years technology innovation of analytical platforms has offered alternative solutions to the ANA-IIF test based on the manual or automated monoplex IMA, mainly of the ELISA type, with the use of solid phases made up by a mixture of nuclear-cytoplasmic antigens. Experience published in the literature has demonstrated that these manual [73 – 84] or automatic [85 – 87] systems do not provide the same analytic accuracy as IIF, in particular for the presence of false negative results (up to 35% of cases) in case of rare autoantibodies [79].

Therefore, it is maintained that the IMA monoplex methods do not represent a substitute for IIF, not even in unusual analytic conditions or when faced with a high volume of test requests [78].

The introduction of multiplex methods (multiplex immunoassays), automated in varying degrees, which are able to simultaneously measure several ANA-related antibodies, has produced – especially in the USA – the hypothesis that they can be used as a screening platform for ANA testing as an alternative to IIF. However, the sensitivity of the ANA screening test with multiplex immunoassay is not yet adequate and the presence of false negative results with respect to IIF is not dissimilar to the occurrence of the previously-discussed immunometric methods [35 , 42 , 56 , 62], varying from 0.2% to 40.5% according to the population studied (Table 4). One of our recent studies conducted with a latest-generation multiplex system (BioPlex 2200) showed a higher sensitivity and a parallel specificity of the ANA-IIF (at 1.80 dilution) compared to the ANA screen on BioPlex 2200. In the study of 181 patients affected by autoimmune rheumatic diseases, the rate of false negatives has stabilized around 11.6% for the manual IIF method, vs. 14.9% for the BioPlex method (Table 5). In most cases, the patients had nucleolar or rare autoantibodies. In a group of 208 healthy and infectious disease patients, the specificity was similar between the two methods (Table 5).

Following the recent statement that the IIF technique should be considered as the standard screening method for the detection of ANA [69], the biomedical industry has proposed technological solutions which significantly improve the automation for the procedure, not only in the preparation of substrates and slides but also in reading under the microscope. This innovation is based on the principles of digitalization of fluoroscopic images and on the classification of patterns using standardized approaches (automatic positive-negative pattern interpretations) using expert

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Patients, n</th>
<th>IIF positive, %</th>
<th>MIA positive, %</th>
<th>Multiplex system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifli et al., 2006 [41]</td>
<td>910</td>
<td>17.3</td>
<td>17.1</td>
<td>AthelNA Multi-lyte</td>
</tr>
<tr>
<td>Bonilla et al., 2007 [42]</td>
<td>53</td>
<td>90.6</td>
<td>49.1</td>
<td>AthelNA Multi-lyte</td>
</tr>
<tr>
<td>Salamunic et al., 2008 [46]</td>
<td>897</td>
<td>27.9</td>
<td>23.6</td>
<td>AthelNA Multi-lyte</td>
</tr>
<tr>
<td>Hanly et al., 2010 [52]</td>
<td>192</td>
<td>81.3</td>
<td>75.5</td>
<td>BioPlex 2200</td>
</tr>
<tr>
<td>Op de Beeck et al., 2012 [65]</td>
<td>236</td>
<td>78.3</td>
<td>74.5</td>
<td>BioPlex 2200</td>
</tr>
</tbody>
</table>

Table 4 Diagnostic sensitivity of indirect immunofluorescence (IIF) and multiplex immunoassay (MIA) methods for ANA testing.

<table>
<thead>
<tr>
<th>Patients</th>
<th>n</th>
<th>IIF positive (1:80)</th>
<th>Bioplex positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>SLE</td>
<td>95</td>
<td>89.5</td>
<td>77</td>
</tr>
<tr>
<td>SSc</td>
<td>55</td>
<td>96.4</td>
<td>52</td>
</tr>
<tr>
<td>SS</td>
<td>18</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>DM/PM</td>
<td>8</td>
<td>50</td>
<td>7</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>181</td>
<td>88.4</td>
<td>154</td>
</tr>
<tr>
<td>Blood donors</td>
<td>120</td>
<td>5.8</td>
<td>6</td>
</tr>
<tr>
<td>Infectious disease</td>
<td>98</td>
<td>7.1</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
<td>6.7</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 5 Sensitivity of methods for ANA detection in AIDs: comparison between IIF and BioPlex 2200.

SLE, systemic lupus erythematosus; SSc, systemic sclerosis; SS, Sjögren’s syndrome; DM/PM, dermatomyositis/polymyositis.
Table 6 Patterns detected by the screening/recognition IIF automated systems.

<table>
<thead>
<tr>
<th>System</th>
<th>Screening −/+</th>
<th>Patterns, n</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aklides</td>
<td>Yes</td>
<td>5</td>
<td>Homogeneous, speckled, nucleolar, centromeric, nuclear dots</td>
</tr>
<tr>
<td>Europattern</td>
<td>Yes</td>
<td>6</td>
<td>Homogeneous, speckled, nucleolar, centromeric, nuclear dots, cytoplasmic</td>
</tr>
<tr>
<td>G-Sight</td>
<td>Yes</td>
<td>5</td>
<td>Homogeneous, speckled, nucleolar, centromeric, nuclear dots, mitochondrial</td>
</tr>
<tr>
<td>Nova-View</td>
<td>Yes</td>
<td>5</td>
<td>Homogeneous, speckled, nucleolar, centromeric, nuclear dots</td>
</tr>
<tr>
<td>Helios</td>
<td>None</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 7 Diagnostic sensitivity of manual and automated IIF methods for ANA testing.

<table>
<thead>
<tr>
<th>System</th>
<th>Patients Manual IIF no. positive (1:80)</th>
<th>Automated IIF no. positive</th>
<th>Agreement, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europattern</td>
<td>116</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Aklides</td>
<td>182</td>
<td>182</td>
<td>180</td>
</tr>
</tbody>
</table>

We evaluated the diagnostic performance of some of these fully automated IIF interpretation systems in routine diagnostics, in comparison with the visual expert interpretation of IIF. A total number of 298 serum samples from patients with suspected autoimmune disease were collected from March to April 2011 for ANA testing in two different experiments, using Aklides (Medipan, Germany) and Europattern (Euroimmun, Germany) systems. Results demonstrated a very high agreement of the two automated systems with the manual IIF method (Table 7).

Our findings are consistent with recent literature data that have shown a high sensitivity (close to 100%) and a good agreement between the Aklides and Europattern systems and visual IIF interpretation for ANA, in patients with AIDs [93, 94].

At present, these test systems might be helpful as a screening tool, although the performance of reading of slides and automated systems needs to be further improved. The availability of these systems suggests that automation of cell-based IIF testing may improve standardization of ANA assessment and help to reduce variability among autoimmunology laboratories in the near future.

However, there is still insufficient evidence that the new automated platforms present the same careful analysis of the IIF patterns as the conventional IIF procedure: this is a critical point, because the recognition of immunofluorescence patterns not associated with systemic autoimmune diseases is helpful in the interpretation of a positive ANA result in healthy individuals [95]. Further studies are needed to define the specificity of automated IIF.

**Conflict of interest statement**

**Authors’ conflict of interest disclosure:** The authors declare that this study was not financially supported by any pharmaceutical organization or industry and that there are no conflicts of interest regarding the publication of this article.

**Research funding:** None declared.

**Employment or leadership:** None declared.

**Honorarium:** None declared.

Received March 27, 2012; accepted May 4, 2012; previously published online July 3, 2012

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