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REVIEW ARTICLE

Automation in indirect immunofluorescence testing: a new step in the evolution of the autoimmunology laboratory

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Abstract Indirect immunofluorescence (IIF) plays an important role in immunological and immunometric assays for detecting and measuring autoantibodies. This technology was the first multiplex method used to detect cardinal autoantibodies for the diagnosis of autoimmune diseases. Over the last 20 years, research has enabled the progressive identification of cell and tissue autoantigens which are the target of autoantibodies originally detected by IIF. Accordingly, newer immunometric methods, capable of measuring concentrations of specific autoantibodies directed against these autoantigens, allowed for a gradual replacement of the IIF method in the autoimmunology laboratory. Currently, IIF remains the method of choice only in selected fields of autoimmune diagnostics. Following the recent statement by the American College of Rheumatology that the IIF technique should be considered as the standard screening method for the detection of ANA, the biomedical industry has developed technological solutions which significantly improve automation of the procedure, not only in the preparation of substrates and slides, but also in microscope reading. This review summarizes the general and specific features of new available commercial systems (Aklides, Medipan; Nova View, Inova; Zenit G Sight, A. Menarini Diagnostics; Europattern, Euroimmun; Helios, Aesku.Diagnostics; Image Navigator, Immuno Concepts; Cytospot, Autoimmun Diagnostika) for automation of the IIF method. The expected advantages of automated IIF are the reduction in frequency of false negative and false positive results, the reduction of intra- and inter-laboratory variability, the improvement of correlation of staining patterns with corresponding autoantibody reactivities, and higher throughput in the laboratory workflow.

Keywords Autoimmune diseases · Indirect immunofluorescence · Automation · Standardization

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Introduction

Indirect immunofluorescence (IIF), a general method for detecting and measuring antibodies and autoantibodies, plays an important role within the immunological and immunometric assays (IMA) (reviewed in [1]). This technology was the first multiplex method used to detect cardinal autoantibodies for the diagnosis of autoimmune diseases (AIDs), beginning with antinuclear antibodies (ANA) in 1957 to endomysium antibodies in 1984 (Table 1) [2–15]. In some applications however, (i.e., for ANA, anti-thyroid antibodies, etc.) IIF is actually a multiplex method, given that it is able to detect from 2 to more than 60 autoantibodies simultaneously; in the case of ANA, it allows the identification of at least 26 different cellular patterns [16]. Thanks to the use of various animal and human tissues (liver, kidney, stomach, esophagus,

Table 1 Milestones of indirect immunofluorescence in the history of autoantibody testing

Autoantibody	Author
Antinuclear	Friou [2]
Anti-thyroglobulin	Nairn [3]
Anti-parietal cells	Taylor [4]
Anti-intercellular substance	Beutner [5]
Anti-mitochondria	Walker [6]
Anti-smooth muscle	Johnson [7]
Anti-adrenal cortex	Irvine [8]
Anti-steroid cells	Anderson [9]
Anti-reticulin	Seah [10]
Anti-liver-kidney microsomes	Rizzetto [11]
Anti-islet cells	Bottazzo [12]
Anti-pituitary cells	Bottazzo [13]
Anti-gliadin	Unsworth [14]
Anti-endomysium	Chorzelski [15]

Table 2 Current use of immunological methods for the detection of autoantibodies in clinical laboratories

Autoantibody	IIF	IMA
ANA	+++	+
Anti-dsDNA	+++	+++
Anti-ENA	–	+++
AMA	+++	+++
PCA	+++	++
ASMA	+++	++
ACA	+++	–/+
APA	++	–
ATA	–	+++
EmA	++	+++
ICA	+	+++
ASA	+++	+
ASCA	+	+++
ANCA	+++	++

IIF indirect immunofluorescence, *IMA* immunometric assays, *ANA* antinuclear antibodies, *ENA* extractable nuclear antibodies, *AMA* anti-mitochondrial antibodies, *PCA* parietal cells antibodies, *ACA* adrenal cortex antibodies, *APA* anti-pituitary antibodies, *ATA* anti-thyroid antibodies, *EmA* anti-endomysial antibodies, *ICA* islet cells antibodies, *ASA* anti-skin antibodies, *ASCA* anti-Saccharomyces cerevisiae antibodies, *ANCA* anti-neutrophil cytoplasmic antibodies

pituitary, pancreas, bladder, nerve tissue, etc.) and isolated cells (human laryngeal HEp-2 carcinoma cells, *Crithidia luciliae* hemoflagellates, human neutrophil granulocytes, fibroblasts, intestinal cells, VSM 47 cells, HEK-239 cells, etc.), IIF has permitted the detection 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 pathologies, according to analytical sensitivity and specificity of the different types of substrates.

The current role of indirect immunofluorescence

Since the turn of the millennium, there has been a rapid advance in diagnostic technology for detection and quantitation of autoantibodies [17]. The reasons for this technological revolution are (a) the increment of awareness of the physiopathogenetic and diagnostic role of autoantibodies in systemic and organ-specific autoimmune diseases; (b) the refinement of procedures for identifying and purifying the target autoantigens of the autoimmune reaction; (c) the application of quantitative immunological methods to automated analytic systems and platforms; and (d) the development of proteomic multiplex technologies, able to detect simultaneously a high number of autoantibodies in the same sample.

Over the last 20 years, basic research has enabled the progressive identification of cell and tissue autoantigens which are the target of autoantibodies originally detected by IIF. Accordingly, immunometric methods capable of measuring concentrations of specific autoantibodies directed against these autoantigens have been introduced, with a progressive replacement of the IIF method in the autoimmunology laboratory [18]. Currently, IIF remains the method of choice in selected fields of autoimmune diagnostics and its relationship with other IMA methods is indicated in Table 2.

The IIF method is now considered the reference method for ANA and anti-neutrophil cytoplasmic antibody (ANCA) screening and a confirmatory test for anti-dsDNA antibody detection [19–22]; other common uses relate to the detection of anti-mitochondria, anti-smooth muscle, anti-adrenal cortex, anti-pituitary, and anti-skin autoantibodies.

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 [23, 24]. Because of these limitations and the progressive increase of autoantibody test requests in autoimmunology laboratories, particularly in the case of ANA, over the last 15 years innovation in the technology of analytical platforms has offered the availability of alternative solutions to the IIF method, based on the manual or automated monoplex IMA (mainly of the ELISA type), with the use of solid phases coated by a mixture of nuclear-cytoplasmic antigens. The literature reports have demonstrated that these manual [25–36] or automated [37–40] 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. Therefore, it is maintained that the IMA monoplex methods

Table 3 General features of automated IIF commercial platforms

System	Company	Neg/pos automated screening	Automated pattern recognition no. (type)
Aklides	Medipan, DE	Yes	7 (homogeneous, speckled, nucleolar, centromeric, nuclear dots, mitotic, cytoplasmic)
Europattern	Euroimmun, DE	Yes	8 (homogeneous, speckled, nucleolar, centromeric, nuclear dots, mitotic, nuclear membrane, cytoplasmic)
Zenit G-Sight	Menarini, IT	Yes	5 (homogeneous, speckled, nucleolar, centromeric, cytoplasmic/mitochondrial)
Nova View	Inova, USA	Yes	5 (homogeneous, speckled, nucleolar, centromeric, nuclear dots)
Helios	Aesku, DE	Yes	None
Image Navigator	Immunoconcepts, USA	Yes	None
CytoSpot	Autoimmun Diagnostika, DE	Yes	None

do not represent a substitute for IIF, not even in unusual analytic conditions or when faced with a high volume of test requests.

The introduction of multiplex methods, able to simultaneously measure several ANA-related antibodies, gave rise to 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 is similar to the previously discussed monoplex IMA methods [1, 41–44], varying from 0.2 to 40.5 % depending on the population studied.

Automation in indirect immunofluorescence

Following the recent statement made by the American College of Rheumatology that the IIF technique should be considered as the standard screening method for the detection of ANA [21], the biomedical industry has proposed technological solutions which significantly improve the automation of the procedure, not only in the preparation of substrates and slides, but also in microscope reading. This innovation is based on the principles of digitalization of fluoroscopic images and on the classification of patterns using standardized approaches (automated positive–negative screening and pattern interpretation). These systems are based on the use of automated microscopes, robotized slide trays, high-sensitivity video cameras, and softwares dedicated to acquisition and analysis of digital images [23, 45–47].

Currently, advanced stages of experimentation are taking place on seven commercial systems (Aklides, Medipan, Berlin, Germany; Nova View, Inova, S. Diego, USA; Zenit G Sight, A. Menarini Diagnostics, Florence, Italy; Europattern, Euroimmun, Luebeck, Germany; Helios, Aesku.Diagnostics, Wendelsheim, Germany; Image Navigator, Immuno Concepts, Sacramento, USA; Cytospot, Autoimmun Diagnostika, Strassberg, Germany). Their characteristics are summarized in Tables 3 and 4.

Types and degree of automation of commercial platforms for indirect immunofluorescence methods

Aklides

The system consists of a fully motorized inverse microscope (Olympus IX81) with a controllable motorized scanning stage, 400/490 nm light-emitting diodes (LED) and a gray level camera. The interpretation system is controlled by the AKLIDES software. Additional di-amidino-2-phenyl-indole (DAPI) stain is used for focusing of objects. Aklides was the first commercially available system. Several recent literature reports show that the system has an excellent diagnostically relevant positive/negative discrimination, with high agreement between automated and visual interpretation: 94.6 % in the study of Hiemann et al. [48] involving 502 samples/subjects; 91–93.0 % in the study of Egerer [49], involving 1,222 patients; 90–95 % in the study of Kivity [50] concerning 397 samples. In a study of our group [51], the overall agreement was 99.0 % (182 samples). 91 % (44 samples), and 89.1 % (46 samples) for ANA, anti-dsDNA, and ANCA testing, respectively.

Nova View

The platform consists of the same hardware of the previous system, with different software. At present, there are no published reports about this system. Preliminary data

Table 4 Specific features of automated IIF commercial platforms

System	Throughput (samples/hour)	Components	Test available	Autofocus with additional fluorescent stain	Specific features
Aklides	48–60	Olympus IX81 Microscope Aklides Software	ANA, DNA, ANCA, LKS	DAPI: di-amidino-2-phenyl-indole	Titer prediction
Nova View	48–60	Olympus IX81 Microscope Nova View Software	ANA	DAPI: di-amidino-2-phenyl-indole	Titer prediction, connection with Quanta-Lyser pipetting station and Modulab Autoimmunity middleware
Europattern	90	Europattern Microscope EuroLabOffice Software (ELO)	ANA	Propidium iodide	Titer prediction, Magazine for automated insertion of slides (500 positions), database with 115,000 images
Zenit G-Sight	14–48	Zenit G-Sight Microscope Zenit G-Sight Software	ANA	None	Titer prediction
Helios	150	Helmed IFA processor Nikon Microscope Helios Software	ANA	None	Full automation
Image Navigator	90	Image Navigator Microscope Image Navigator Software	ANA, ANCA, DNAAb, LKS	None	Connection with AFT 3000 pipetting station
Cytospot	96	Cytospot Microscope CytoSpot Software	ANA	None	Full automation, high throughput robot (100 slides)

demonstrate that the correlation between the Nova View system and the conventional method ranges from 93–100 %, in the classification of negative–positive samples and in interpretation of fluorescence pattern. In a recent unpublished study by our group, we were able to confirm these data, with agreement between the Nova View System and the manual IIF of 95 %.

Europattern

The instrument consists of a fully motorized microscope that allows the automated processing of up to 500 analysis positions in succession with identification of pattern combinations (including the corresponding titers). The interpretation system is controlled by the EuroLabOffice (ELO) Software, with a reference database containing 115,000 images of samples. In a recent study of our group (unpublished data) aimed to validate this new system, we examined 116 unselected sera from outpatients. The agreement between the automated and manual classification of these sera was 100 % in the case of positive sera and 74 % in the case of negative sera: the overall concordance was 94 %.

Zenit G-Sight

The system consists of a motorized automated fluorescence microscope that allows a semi-quantitative analysis (with different G-Sight indexes) and intelligent pattern recognition for ANA test by the use of a specific software. Published reports are not yet available on the validation of the system. In preliminary studies of different researchers, the system demonstrates a good correlation between the quantitative estimation of ANA intensity and manual ANA titers as assessed by serial dilution and shows a sensitivity of 64 % and a specificity of 97 % at index 24, with a concordance between index-based and validated results of 96 % at index <14. In a recent study of our group (preliminary data), we were able to confirm these results, but we found a high percentage of uncertain results.

Helios

The system was developed based on the IIF processor Helmed. An optical system to capture the respective IIF images was implemented and a specific software algorithm was developed for the differentiation between positive and negative samples; the results can be recorded, saved and transmitted to the laboratory information system and validated remotely. The in-house validation of the system with over 1,000 serum samples showed a 98.4 % correlation of results, when compared to the manual IIF procedure and visual interpretation.

Image Navigator

The system consists of a motorized fluorescence microscope that automates the scanning of patient samples on Immuno Concepts fluorescent HEp-2000 assay by capturing up to four images per field, and sorts them into positive and negative folders for review by the technologist. Unpublished data show a high between-reader agreement (97 %).

Cytospot

The system consists of fully automated diagnostic microscope for immunofluorescent images that scans all conventional slides and allows a stand-alone differentiation of positive and negative samples and a guided comparison of the output image with images in the library of integrated ANA patterns. For high throughput laboratories, a second version of the system exists (Cytospot Robot) that allows the automated imaging of 100 slides. Currently, the literature reports are not available on the diagnostic accuracy of the system.

Advantages and perspectives of automated IIF cell/tissue assay

Automation of the IIF method can be used for a cost-effective and accurate screening for diagnostically relevant autoantibodies; this technology may play down errors and problems caused by subjective image evaluation and low expertise. The further development of classification algorithms should allow the identification and differentiation of a wide variety of cell and tissue staining patterns.

The expected advantages of an automated IIF method are (a) the reduction in frequency of false negative and false positive results due to the standardization of measurement of fluorescence intensity; (b) the reduction of intra- and inter-laboratory variability; (c) improvement in the correlation of staining patterns with corresponding autoantibody reactivities; and (d) higher throughput in the laboratory workflow.

Conflict of interest None.

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