RESEARCH UPDATES

Zenit PRO: Towards Total Automation and IIF Standardization

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System overview

Analysis of autoantibodies by indirect immunofluorescence (IIF) remains the hallmark of autoimmune disease diagnosis. This technology was the first "multiplex" method used to detect key autoantibodies: in the case of ANA, in fact, IIF on HEp-2 cells allows the identification of at least 28 different cellular patterns correlated to more than 60 autoantibodies[1].

Automation solutions of the IIF method have been developed to improve laboratory workflows and ensure cost-effective and more accurate screening for diagnostically relevant autoantibodies by reducing errors caused by several manual operations and subjective image evaluation.

The newly developed Zenit PRO system is a fully automated solution for autoimmune laboratories performing IIF assays, which streamlines the complete IIF protocol from slide processing to reading and interpretation of results. The project stems from the need to improve IIF standardization protocols, to reduce costs and increase the efficiency, productivity and quality of laboratory operations, with a remarkable impact on overall laboratory management.

Zenit PRO integrates an automated slide-processing module with a reading unit. The system automatically processes and aids in the interpretation of IIF tests and advanced dedicated software orchestrates multiple processes into a seamless system (Figure 1).

The technology used to process and read the slides is tailored to the requirements of the IIF technique. The system

Figure 1: Zenit PRO – the system integrates an automated slide-processing module with a reading unit. An advanced software orchestrates multiple processes into a seamless system

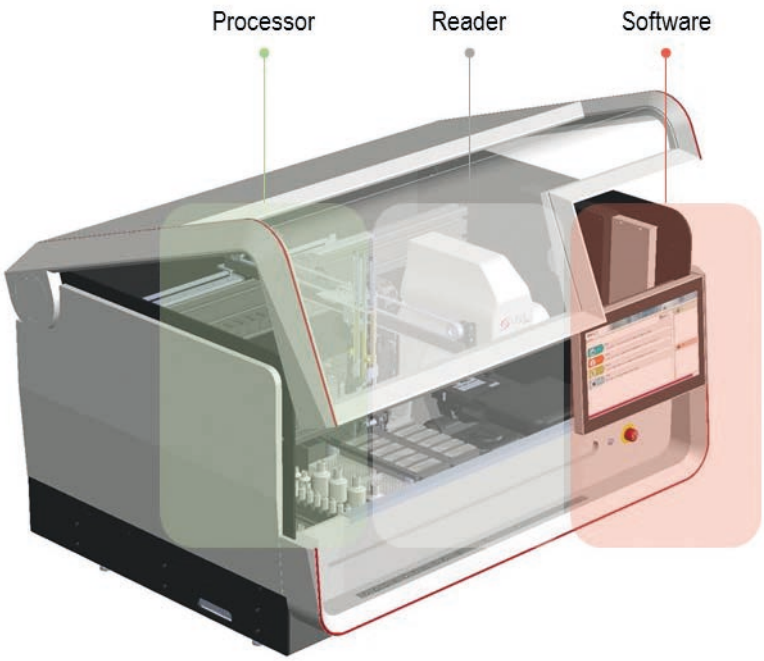
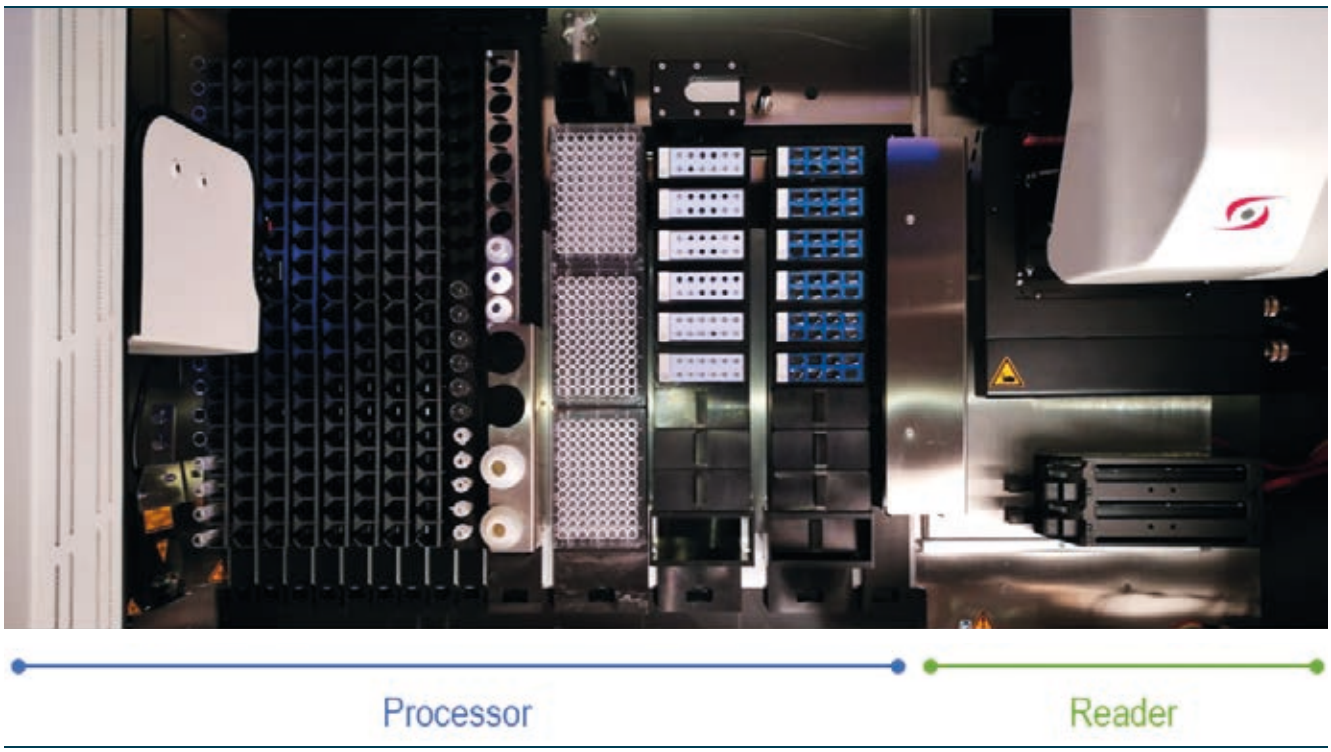


Figure 2: Zenit PRO Workplan – the system is able to prepare, scan and read 18 different slides in a single run



comprises a liquid-handling robotic unit designed for slide processing and a motorized microscope unit for image acquisition and whole well scanning. The liquid handling system aspirates and dispenses samples, reagents, controls, washing solutions and the mounting medium. The system processes 18 slides in a batch and opens up the possibility of "continuous access" to add further tests while the system is operating. The software automatically schedules and organizes the steps to ensure uniform incubation times across each slide. After the incubation and washing procedures, the system automatically mounts each processed slide: the mounting medium is carefully dispensed over the slide and a dedicated tool joined into the sampling arm places the coverslip to cover and seal the slide avoiding bubble formation. After the mounting procedure, each slide is delivered onto the microscope precision stage for automatic scanning of each well of the slide. The motorized microscope drives to the substrate positions, autofocuses and scans a square area inside the rim of each well. The digitized image is then displayed and can be navigated with the virtual microscope tool that allows the user to have a broad view of the substrate at multiple magnifications. After termination of the reading procedure, a precision clamp delivers the slide into a slide parking rack for slide disposal (Figure 2).

The system can process and scan various cellular substrates, including HEp-2, neutrophils and *Crithidia luciliae* as well as a variety of tissues such as liver, kidney, stomach and monkey esophagus. Results are interpreted on screen by the user, who can classify and report each test result from a powerful yet intuitive software interface.

The system includes an automatic classification of positive/negative results for ANA tests and identification of a number of cellular patterns (nuclear and cytoplasmic patterns including mitotic figures) even in mixed cases. The software measures the intensity of fluorescence for each positive test and provides a titer suggestion based on a wide database of reference images used to train a state-of-the-art classifier. The software for automated determination of positive/negative results is designed to efficiently classify ANCA/c-ANCA, nDNA and EMA tests as well.

The system orchestrates multiple processes in order to simplify the workflow, increase the safety of IIF testing by full traceability and to minimize technologist interaction all along a high-volume testing procedure.

Major benefits of a fully automated IIF system:

- Streamlined process for simple operation
- End-to-end management of overall IFA protocol
- Standardization of IFA protocol
- Harmonization of results

Table 1: Automatic interpretation performed by different systems on the market

Test	Interpretation	NovaView	Europattern	Image navigation	Aklides	Helios	G-Sight
ANA	Pos/Neg	Yes	Yes	Yes	Yes	Yes	Yes
	Pattern recognition	Yes	Yes	-	Yes	-	Yes
	Titer prediction	Yes	Yes	-	Yes	-	-
ANCA	Pos/Neg	-	-	-	Yes	-	Yes
	Pattern recognition	-	-	-	Yes	-	Yes
DNA	Pos/Neg	-	-	-	Yes	-	-

- Reduction of intra- and inter-laboratory variability
- Reduction of operating costs

State of the art

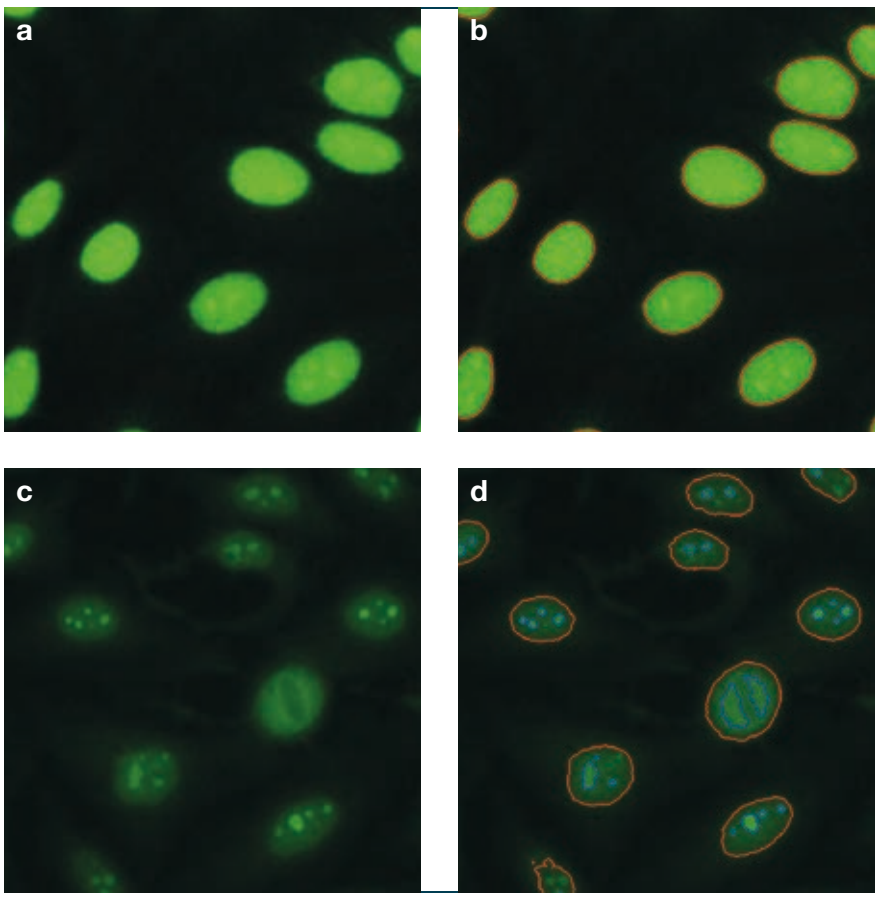
A few automated slide processors are currently available on the market, IF Sprinter by Euroimmun, Quanta-Lyser by Inova Diagnostics and HelMed by Aesku being just some examples. These systems prepare slides for manual mounting, and after that critical step the slides can be read and possibly interpreted with a manual or semi-automated microscope.

In recent years, automated slide reading and interpretation systems have been also developed and introduced. Both NOVA View of Inova Diagnostics, Europattern of Euroimmune and Image Navigator of Immunoconcepts follow the same steps: they acquire a certain number of images per well in specific areas and provide an interpretation of results[2-6].

Table 1 summarizes the list of tests automatically interpreted by each of these systems. Regarding ANA HEp-2, all devices are able to discriminate between positive and negative samples, some of them can detect one or more HEp-2 patterns, and some are able to interpret positive/negative results of ANCA and nDNA tests. Europattern of Euroimmune also performs a titer prediction that requires at least two dilutions in the screening step, making the procedure not particularly cost saving. Helios, manufactured by Aesku, is the only system integrating automated slide preparation with slide reading thanks to the presence of an integrated microscope and camera that captures a limited number of images.

Nevertheless, whenever the user is not confident with the automated results (because the number of acquired images is not enough to provide the whole picture), evaluation of the slide under a manual mi-

Figure 3: (a) Homogeneous detail image; (b) Fluorescence target detection on cells displaying a homogeneous pattern; (c) Nucleolar detail image; (d) Fluorescence target detection of nucleolar stained cells (blue profile)



croscope still remains a necessary step to complete the test.

In 2010 Visia Imaging presented a new concept to perform automated reading and interpretation of IFA tests and introduced the Zenit G-Sight system in the market. This system performs the complete digitization of the well generated by a mosaic of single images that can be navigated through a dedicated software tool called Virtual Microscope at different magnifications. Zenit G-Sight can discriminate between positive and negative samples and can give a pattern suggestion for both ANA HEp-2 and ANCA tests[7-8] (Table 1).

ZENIT PRO test interpretation

The Zenit G-Sight algorithms implemented for automated interpretation and classification of IIF test results on HEp-2 and neutrophils have been widely improved in the Zenit PRO system. Moreover, novel algorithms for positive/negative classification of nDNA and EMA tests have been implemented.

Below are listed the main features of the new software for the automated interpretation of IIF results:

1. **ANA Positive/Negative classification**
   An evaluation of fluorescence intensity is performed on every single identified cell. An average level of fluorescence is calculated according to the fluorescent structures detected inside each analyzed cell. As shown in Figure 3a and 3b, cells displaying a homogeneous pattern have a homogeneous level of fluorescence, whereas for cells displaying nucleolar staining (Figure 3c and 3d) the analysis of fluorescence is limited to the nucleolar structures. This response – according to the titration and the level of fluorescence obtained – is also calculated for other types of patterns, and two thresholds are used to classify the results into three different classes: negative, borderline and positive. Zenit PRO software displays an index value representing the level of fluorescence that gives an indication on the positivity of the sample analyzed.
2. **Recognition of 9 HEp-2 patterns** (homogeneous, fine speckled, coarse speckled, nucleolar, centromere, mitochondrial, ribosomal, few nuclear dots, multiple nuclear dots).
   The first step in the image processing algorithm consists in the use of morphological operators and threshold techniques to separate background from foreground. The segmentation of foreground is performed in order to evaluate each single cell. In a second step, a collection of texture features are analyzed to evaluate the intensity surface of the cells. Finally, a supervised learning classifier is used to classify patterns using the descriptors. Mitotic figures of recognized patterns are properly detected and shown in a dedicated gallery of the software interface and each figure can be easily identified in the digital well, automatically relocated at the center of the screen.
   In Figure 4a and 4c, two images of a speckled and a nuclear dots pattern are shown. In Figure 4b and 4d the results of cell segmentation and classification of each analyzed cell are displayed. For both patterns a pseudo-color is used to classify the cell as speckled (purple) or nuclear dots (cyan).
3. **Recognition of 3 ANCA patterns** (p-ANCA, c-ANCA, *other*-ANCA and Negative).

Figure 4: (a) Image of speckled pattern; (b) Classification of speckled image; (c) Image of multiple nuclear dots; (d) Classification of multiple nuclear dots image

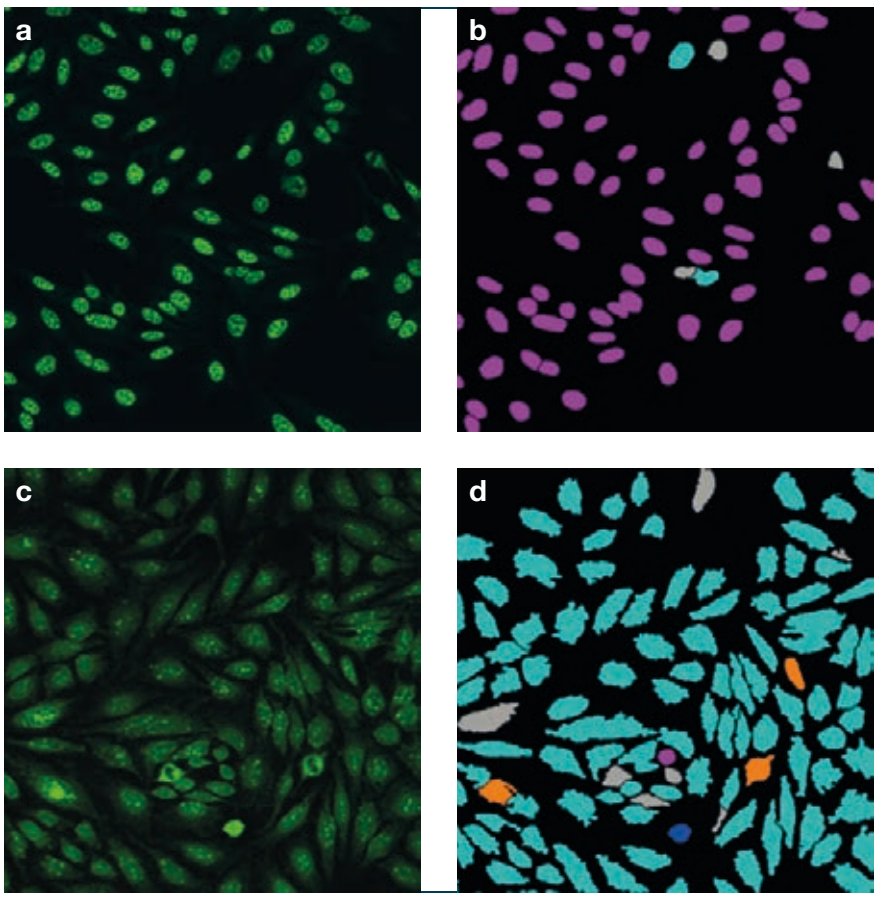
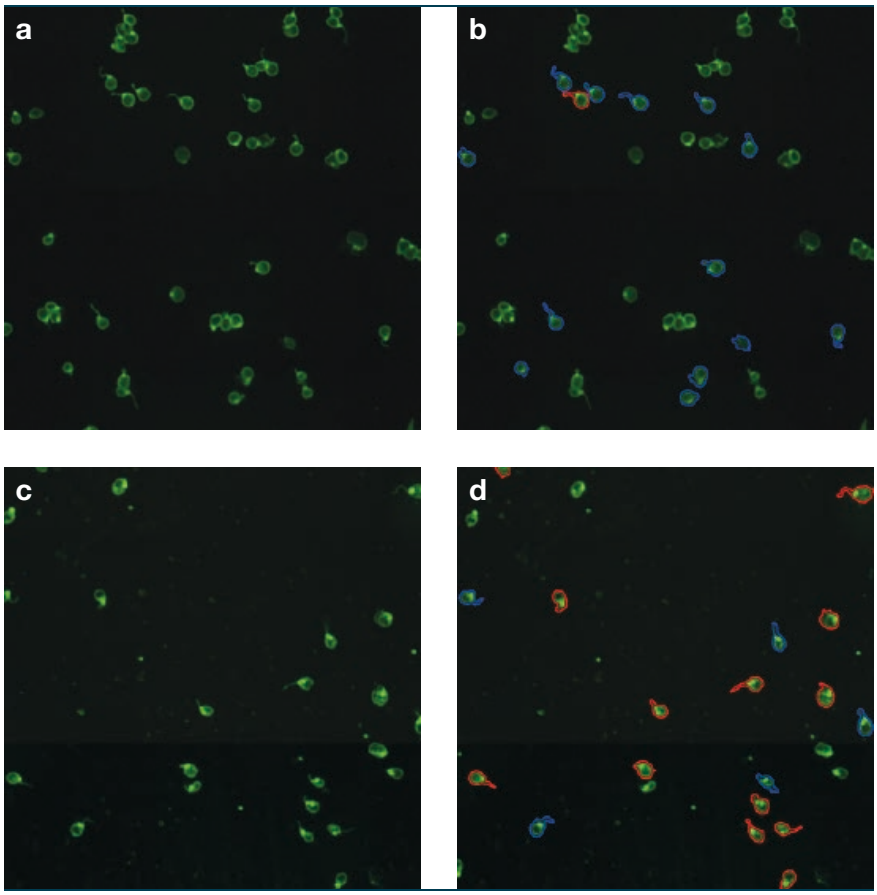


Figure 5: (a) Negative DNA image; (b) Detection of negative particles (blue outline); (c) Positive nDNA image; (d) Detection of positive particles (red outline)



At the basis of the analysis is the identification of the cells through separation of the background from the foreground and cell segmentation whenever clusters are present. For each cell, a collection of features is calculated and used by the classifier to provide a pattern suggestion. Four are the classes of interest: p-ANCA, c-ANCA, Negative and Other. A single training phase is requested for ANCA Ethanol (p-ANCA, c-ANCA, Negative and Other) and ANCA Formalin (c-Anca, Negative and Other) to optimize the classification capability.

4. **Positive/negative classification of nDNA tests on *Crithidia luciliae*.**

The discrimination of positivity is based on the detection of the hemoflagellate organisms as the first step. Then, fluorescent particles related to the basal body, the kinetoplast and the nucleus are analyzed. The presence of basal body is detected taking the flagellum as a reference point, whereas the kinetoplast and the nucleus are analyzed – if present – in terms of average fluorescence intensity. The system displays an indication of positivity (Figure 5) and a measurement of the mean intensity of the kinetoplast.

5. **Positive/negative classification of EMA test.**

For EMA testing on monkey esophagus, the morphology and the intensity of a consistent and specific part of the tissue has to be analyzed. The fixed tissue section on Zenit EMA slides has a typical arrangement which is shown in Figure 6a. In Figure 6a, a positive sample and a control case are shown. The tissue is arranged as a tubular cross-section where the inner part presents an empty area (excluding the membrane). Tissue detection begins with the identification and the removal of the central "empty" (non-informative) zone, highlighted in red in Figure 6b and 6d, and then through iterative evaluations of the variations in average fluorescence intensity. Whenever there are positive peaks or negative peaks, the inner part of the tissue is detected and a thin profile of the area of interest is extracted (blue outline in Figure 6b and 6d). Finally, the average intensity is evaluated at high magnification and target tissue recognition is performed to evaluate if the sample is positive or negative.

For each of the above tests the system performs the analysis on the whole well and not just on a limited number of pre-defined images only partially covering the area of interest.

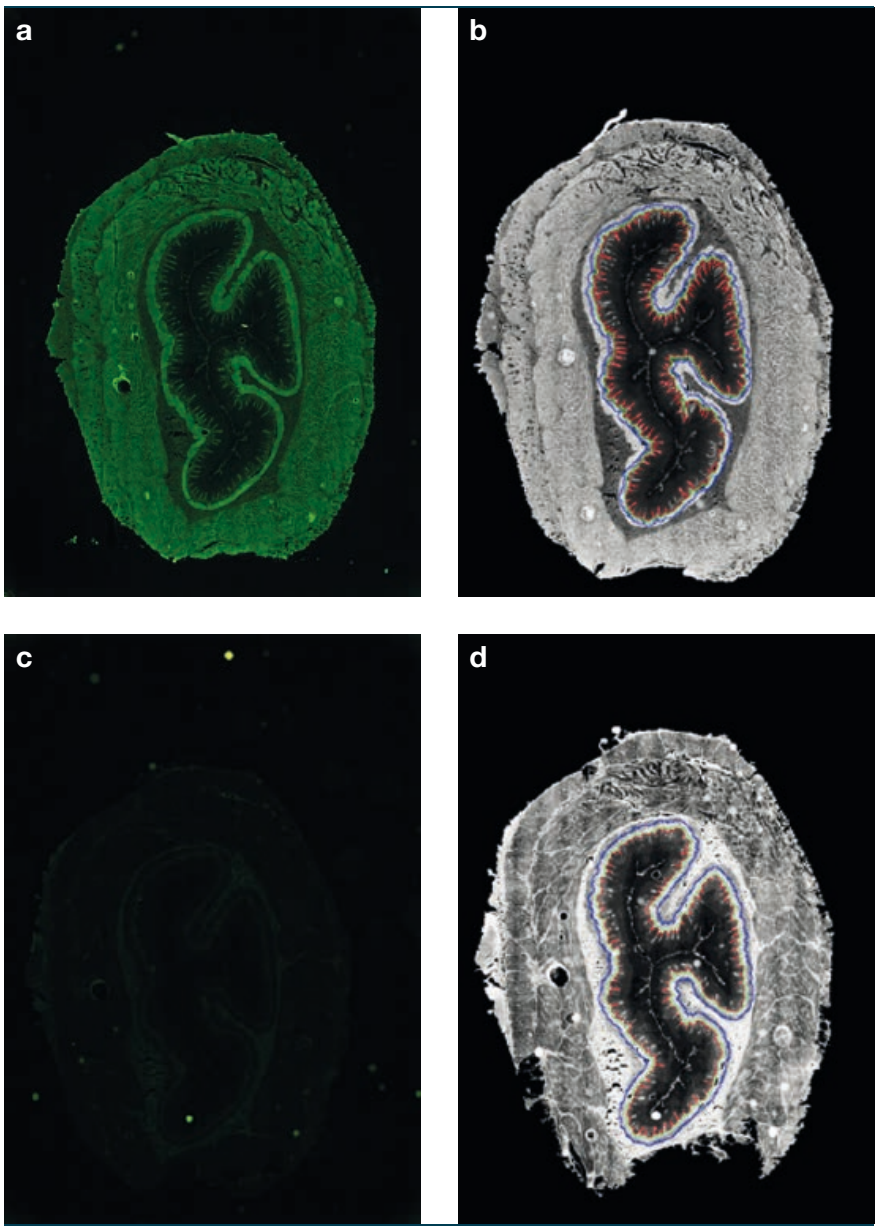
This feature offers several advantages:

- As the number of counted and analyzed cells is fairly large (greater than 3000 for each well), the results are more reliable and consistent;
- Results are robust even in the presence of microbubbles or damaged areas in the well;
- High sensitivity due to the possibility to adjust the cell count and cell identification to detect rare patterns or to detect a pattern displayed by a few cells only.

Conclusion

A. Menarini Diagnostics' partnership with Visia Imaging dates back to 2010, when Zenit G-Sight reader was first introduced in the market.

Figure 6: (a) Low magnification view of EMA-positive control; (b) Enhanced image view and detection of target tissue to be evaluated (blue outline); (c) Low magnification view of EMA-negative control; (d) Enhanced image view and detection of target tissue (blue outline)



The continuous efforts to strive for some form of standardization in a very subjective field such as Autoimmunity led the two Companies to join forces in developing, producing and distributing an automated reader in IIF. At that time, the Zenit G-Sight represented a novelty in the Autoimmunity field because manual microscopy was still considered the golden standard in IIF reading and interpretation.

After some years – during which a number of automated systems have been introduced in the market – things have changed and nowadays automated readers are quite well accepted for reading and interpreting IIF slides even by those who were initially skeptical. Many steps forward have been made over the years to make these systems increasingly reliable, reach sensitivity and specificity values in line with recommendations, include pattern suggestions among the different capabilities, obtain reproducible results, and guarantee analytic precision.

Thanks to the success obtained over the years with the Zenit G-Sight, the A. Menarini Diagnostics and Visia Imaging partnership has become even stronger in a continuous research for new technologies to be used for cost-effective screening of relevant autoantibodies, reducing intra- and inter-laboratory variability and eliminating errors caused by subjective manual preparation and evaluation of IIF slides.

Zenit PRO is the result of such a search, representing a third-generation system where not only reading and interpretation are performed automatically but where also slide preparation – including the mounting phase – is totally automatized thereby lessening the effects of inadequately expert personnel or possible differences among laboratories across Europe.

Autoimmunity has in fact always been burdened by some unfavorable aspects, mostly related to the need to have expert operators and the critical consequences of subjective interpretation but most of all to the lack of any real standardization.

A first step was made many years ago by automatizing the preparation phase, a second one has only recently been made by rendering the reading phase automatic.

It's now time for a real change to complete this standardization process and Zenit PRO is the solution.

A new way to harmonize results, guarantee full traceability and make life in the laboratories easier.

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