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Fluoresce *In-Situ* Hybridization Automatic Analysis Applications

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Abstract

The most evident evidence of the power and flexibility of a diagnostic tool is its range of applications. Non-automated FISH analysis has clearly become an essential tool for clinical practice over the past decade. At the same time, automatic analysis is gaining ground, which is evidenced by the increase in published articles each year on both technical topics and the flexibility of this technology. At the same time, more and more studies are being published that deal with the superiority of automatic analysis over non-automatic analysis. In this review, some pioneering applications of automatic FISH image analysis are described.

Pioneer Applications

First papers on the automated evaluation of FISH signals were published in the early 90s independently by two scientific groups [1,2]. Cremer et al. [1] analyzed 131 previously digitized images of female human lymphocytes characterized using a pXBR probe, which detects the centromere of the X chromosome. Nuclei were selected by eye for digitization based on clear FISH signals. Corrected classification rates in normal and pathological cells were found to be 90% and 85% using META 4 and META 5 programs, respectively. These programs are intended for automatic analysis of digital images. The gray level and the area measured within a single channel were considered recognition signals. Among other things, they concluded that digitization should be done directly from the sample and nuclear boundaries should be determined before starting signal segmentation.

Nederlof et al. [2] used peripheral blood lymphocytes to test the ability of the Tool Command Language (TCL) image system to quantify FISH signals. In addition to the counter stain 4'6-Diamidino-2-Phenylindole (DAPI), the regions around the centromere of chromosomes 1 and 7 were labeled with the dyes Allyl Isothiocyanate (FITC) or Tetramethylrhodamine Isothiocyanate (TRITC). Exposure times for each slide and fluorophore were determined empirically aiming for maximum gray-level resolution. Separation of nucleus DAPI staining from background was performed by histogram-based segmentation. Data from incorrectly segmented nuclei or FISH spots, and additional nuclei with out-of-focus FISH spots were manually excluded. The image analysis process was automated without user interaction and 95% accuracy was achieved. The major problems were caused by heterogeneous backgrounds of nuclei and limited DOFs which were addressed by digital filtering and the use of objectives with lower numerical apertures, respectively.

These ground-breaking initiatives started a far-reaching technical revolution by attracting various experts in the field with different basic science backgrounds. Physicists, biologists, chemists and mathematicians worked together in this untapped field, focusing mainly on key areas such as optics, biostatistics and digital topology. In fact, many early studies did not yet aim to compare their results with those of manual analysis, but instead to investigate various mathematical morphology approaches for optimal object recognition, with a comparative bias. Although rapid technological and computational progress has been achieved, tools and software have been developed in a non-harmonized manner.

In the mid-1990s, Tanke et al. [3], reported that hardware development preceded software development, therefore efforts should be made to improve the latter, especially in terms of the processing of multivariate data. Subsequently, for a short period of time, the focus was rather on automatic in situ bright field fluorescence, and several developments including the automatic development of histological specimens were actually achieved by investigating non-fluorescent specimens [4]. These techniques involve in situ hybridization but without fluorescence. In most cases, chromogenic substances were used. Chromogenic *in situ* Fluorescence (CISH) could be a

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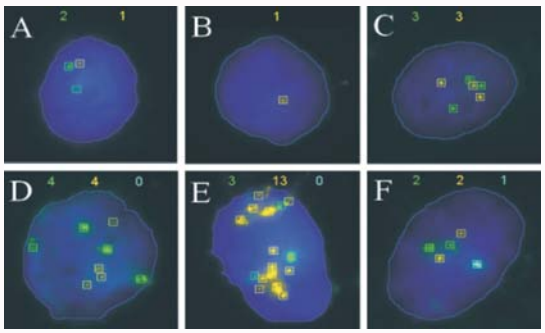


Figure 1: Image analysis [59].

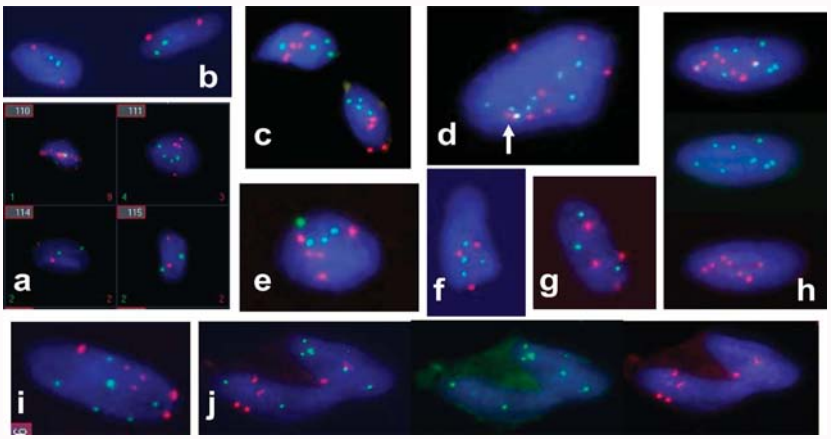


Figure 2: Polyploeidia.

method of choice when a fluorescence microscope is not available and only one or two different probes are applied. Although, even today, CISH is still a special technique, the main attention quickly turned to fluorescence applications.

In 1997, two landmark studies were published on automated FISH analysis by the group of Netten et al. [5,6]. These studies often still serve as benchmarks for system development because they provide a comprehensive and detailed description of each step of point counting. In addition, they pose critical issues for sample quality analysis, such as low cell density, inhomogeneous cell distribution, signal separation and overlap.

By the end of the 90s, Adiga et al. [7] made the first attempt to analyze FISH labeled tissue sections in a 3D image. They quantified CEP7 signals in 10 lm tob15 lm thick tissue sections from prostate cancer patients using confocal microscopy and semi-automated image analysis and reported automatic counting results almost comparable to those of visual counting [8].

Along with technological development, it has been recognized that sample quality has a huge impact on the performance of automated systems, so various efforts have been made to achieve brighter FISH signals using various signal amplification methods [9].

Apart from the first technical comparative studies, results of automatic analysis were almost always subjected to comparison with results of non-automatic analysis considering the latter one as a benchmark. The correlation between the two assessment methods was assessed several times, and eventually led to the introduction of statistical methods and the mathematical correction (reduction) of systematic errors. Ortiz de Solórzano et al. [10] tested the Castleman's correction method and it was found to be useful for improving the performance of an automatic system. Furthermore, they concluded that no sample-based correction is necessary if a sufficiently high number of cells, prepared in a standardized manner, is used for the correction.

Range of Applications

While the technological development and evolution of Information Technology continued unabated in the following years [11-31], there was even more emphasis on practical approaches and a wide range of clinical applications emerged in the early 21st century.

In the field of cancer cytogenetics, automated investigation of HER2 gene amplification has been the most frequently published application, performed mainly on breast cancer samples [23,28,30,32-40]. With the introduction of new targeted chemotherapy treatments, accurate assessment of HER2 gene amplification, such as the ratio of HER2 spots/points and centromeric signals on chromosome 17, has become important especially in the case of patients with a weakly positive [7] HER2 gene. Signals that do not have the form of a spot or signals that are in contact or overlapping make non-automatic analysis tedious and difficult which is probably the reason why many groups have shown special interest in automatic and quantitative analysis of HER2 FISH images.

Rygiel et al. [41] studied using automated FISH analysis the status of the HER2 gene in swab cytology from esophageal patients and found perfect agreement with the results of manual analysis [41]. Image analysis from an esophageal mucosa sample is shown in

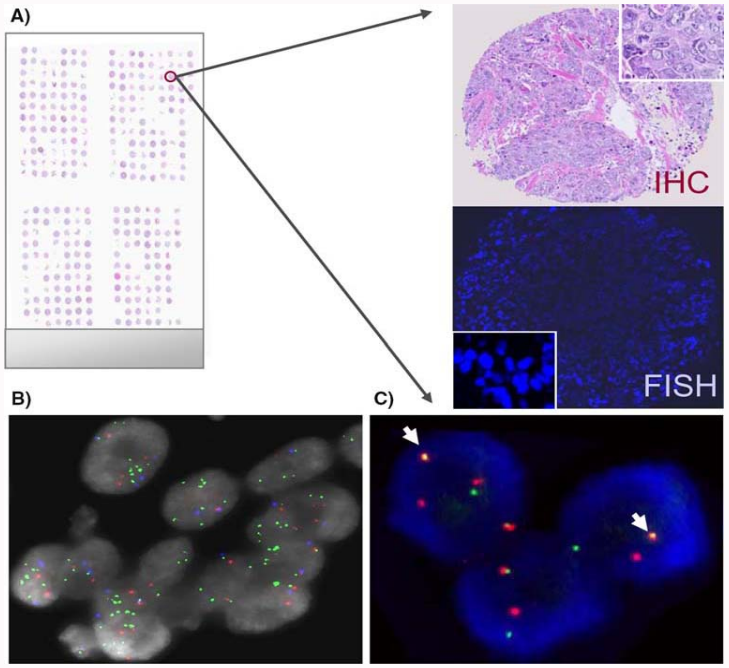


Figure 3: FISH on a microarray sample.

Figure 1. Image F shows the nucleus with normal signals, while the others show nuclei with numerical disturbances. More specifically in images A and B we have a lack of chromosomal material while in images C, D and E we have trisomy (chromosome tripling), tetrasomy (chromosome quadrupling) and polyploidy (the orange signal appears more than eight times) respectively.

In addition to the HER2 gene, automated analysis has also been used to detect various genetic abnormalities in solid tumors. Truong et al. [42] detected chromosome 1 instabilities in breast cancer cells and chromosome 3 instabilities in lung tumors using probes specific for the respective chromosomal arms.

Adiga and Chaudhuri [7] identified a trisomy of chromosome 7 in tissue samples from patients with prostate cancer, an aberration known to be associated with disease progression. Hruska et al. [43] studied three different types of peripheral nerve sheath tumors using centriomeridal probes for chromosomes 7, 17, and 18 and observed different proportions of cells with anosmia in benign and malignant tumors.

From this study, examples with polyploidy, i.e., an increase in chromosome numbers, are shown in Figure 2.

Brown and Huntsman examined tissue microarray slides containing ovarian and breast tumor samples using trichrome probes for the EMSY genes, CCND1, and the centromeric region of chromosome 11. The EMSY gene is associated with poor prognosis and has been found in 13% of breast cancer cases. Breast [44].

An example of FISH on a microarray sample is shown in Figure 3. Figure 3B shows the amplification of the gene with the green signal relative to red and blue. In section C, translocation t (12;15) (p13;q26) is shown by the arrows.

Pajor et al. [45-47] studied aneusomies of chromosomes 3, 7 and 17 and lack of chromosome 16 (9p 21) in urethral epithelial cells of bladder cancer patients using a multicolor approach. It was shown that the ratio of FISH positive cells correlates with both the grade and the stage of the disease, thus the method provides an alternative and efficient way to non-invasively define these characteristics of the specific tumor. The same method was used by Marganski et al. [48] examining 3,000 cases [48].

Seppo et al. [49] investigated duplication of the 3q26 region in low-grade squamous intraepithelial lesion of the cervix, demonstrating its association with an increased likelihood of progression to high-grade dysplasia.

Katz et al. [50] studied 3p22.1 and 10q22.3 abnormalities in sputum samples from lung cancer patients and high-risk smokers. Chromosomal abnormalities were seen in both normal and atypical sputum epithelial cells confirming that cellular genetic abnormalities of 3p22.1 and 10q22.3 reflect a domain of oncogenetic effect within the bronchial cells of individuals at high risk for cancer development.

Hieber et al. [51] investigated cell cultures from papillary thyroid cancers for the presence of specific gene recombination's. In Figure 4 we observe in the left part normal cells from a thyroid gland with a nucleus with two green and two red signals. In the right part we observe a separation of one of the two fusions therefore we have a displacement of genetic material.

Wang et al. [31] examined monosomies and polysomes of chromosomes 3 and X in Pap-Test samples from patients diagnosed with cervical cancer. They concluded that the automated method showed a high correlation with the non-automated one and can be an effective tool to detect cervical cancer at an early stage and predict the progression of the disease. Figure 5 shows from this study the analysis of four cores, their segmentation and the final point count result in a Table.

Balanced genetic aberrations in oncological and hematological diseases represent a unique challenge for automated systems. Chronic myelogenous leukemia and BCR/ABL1 chimeric protein was the beginning of the published work with first Kozubek et al. [52], and

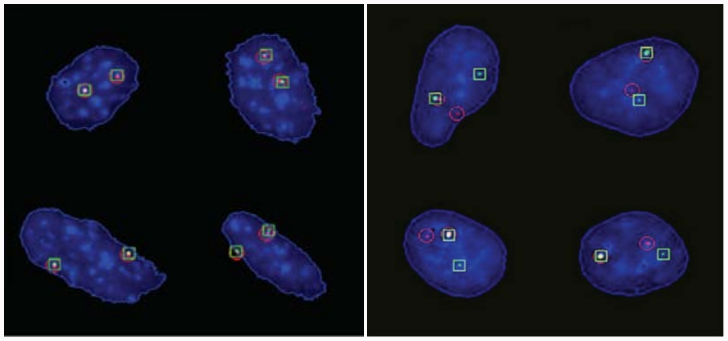
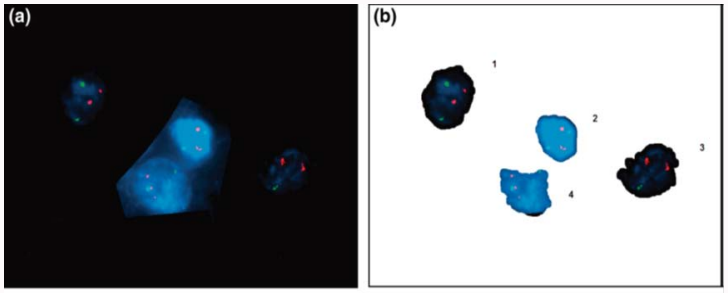


Figure 4: Normal and abnormal cells from a thyroid gland.



(c)

Cell	Type	Red Spots	Green Spots
1	Normal	2	2
2	Normal	2	2
3	Abnormal	2	1
4	Abnormal	3	2

Figure 5: Analysis of cell from cervical cancer.

Lukasova et al. [19] recommending a method for this purpose and publishing a detailed description for the automated evaluation [19,52]. Due to the development of translocation probes to detect BCR/ABL1 positive cells even when they are present in a very small proportion, the method is nowadays a key part of the therapeutic response and monitoring of patients with chronic myelogenous leukemia [53,54].

Reichard et al. [55] and Alpár et al. [56] examined various immunoglobulin heavy chain gene mutations in whole tissue samples from patients with B-cell non-Hodgkin lymphomas. Despite the fact that different sampling methods (tile *vs.* grid sampling) were applied, both groups were able to distinguish positive from negative neoplastic samples. Figure 6 shows a normal image of a healthy lymph node (from two red and green signals), an image of a lymph node with mantle lymphoma (one normal pair and one with gene fusion due to reciprocal translocation t (11;14) (q13;q32)) and an image Burkitt's lymphoma (again a normal pair and a t (8;14) (q24;q32) fusion result).

Numerical chromosomal abnormalities have also been analyzed in hematopoietic tissue disorders. Blandin et al. [57] studied polysomes of chromosomes 4, 6, 10 and 17 for acute lymphoblastic leukemia with a hyperploid karyotype using tetrachrome FISH. The large number of nuclei labeled revealed a high level of chromosomal variability both at diagnosis and at relapse, which may have prognostic significance and a huge impact on future therapeutic strategies. In Figure 7 we observe examples of polyploidy from lymphoblasts of this particular study.

Automated analysis is also very useful in non-oncological cases because the samples may have a much lower cell density (e.g., amniotic fluid investigation) than in the case of cancer samples or when analysis of a large number of cells is required (e.g., investigation of human spermatozoa). These conditions make manual analysis extremely time-consuming and tedious. The first study on automated detection of aneusomy in human sperm was published by Baumgartner et al. [12]. Sperm cells on smears were labeled with LSI13 and CEPX probes and examined with a proven system managing to reduce analysis time from 8 h/smear to 30 min/smear. They concluded that the automatic laser scanning system is able to replace non-automatic analysis.

Later Perry et al. [58,59] extended this application by introducing a system capable of using three fluorochromes (CEPX, Y, and 18) simultaneously in order to determine the frequency of disomy in race chromosomes and reached the same conclusion as the previous study. Likewise, Carrell et al. [13] also investigated sperm cells using probes for five chromosomes (13, 18, 21, X, and Y) and also found that automated systems significantly reduced analysis time. In addition, a high degree of reproducibility has been demonstrated by investigating samples from patients with infertility and tetraploid.

A mesosomes were identified in cells in the amniotic fluid by several scientific groups, achieving significant progress in prenatal screening [29,60-63]. In Figure 8 we see automatic detection of trisomy 21 (three yellow dots) with normal chromosome 18 (two green dots) in an amniotic fluid sample.

Automated FISH analysis has also been applied to non-invasive approaches to prenatal testing, namely, the analysis of fetal cells derived from the cervix [64] or collected from maternal blood [17,65]. To improve the efficiency of detecting fetal cells that are rarely found in maternal blood, Seppo et al. [66] and Oosterwijk et

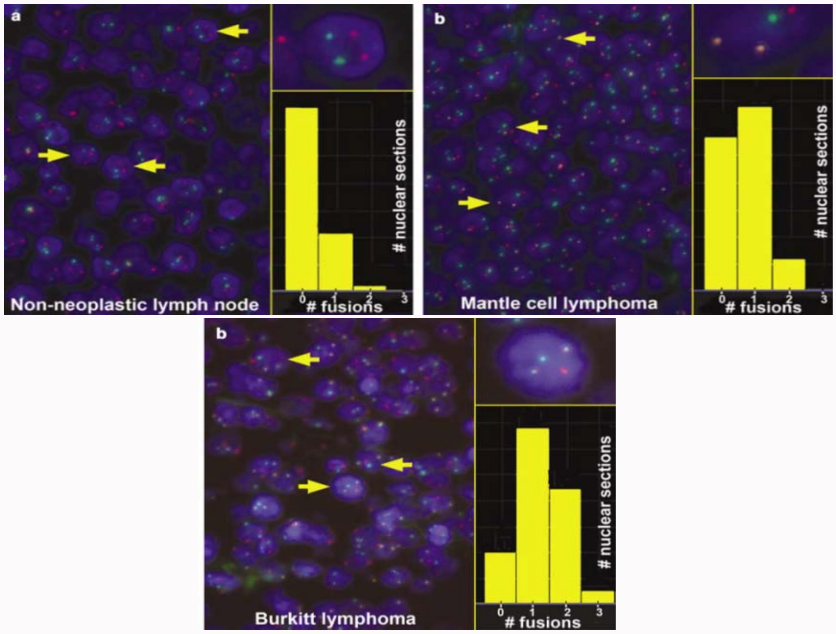


Figure 6: Analysis of samples from B-cell lymphomas.

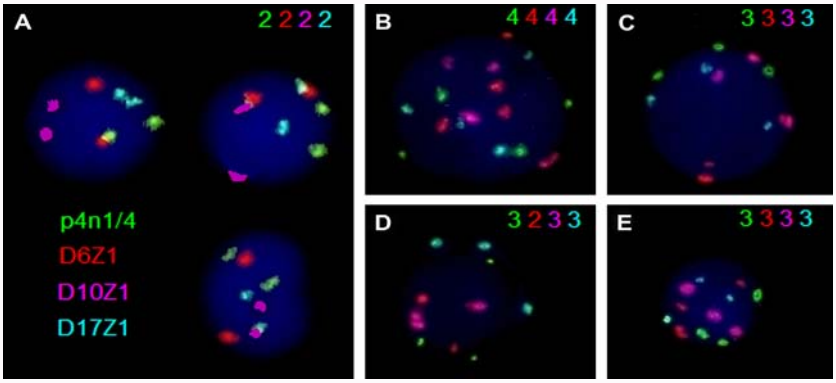


Figure 7: Examples of lymphoblasts.

al. [67] introduced an immunophenotypic method based on fetal hemoglobin staining.

In Figure 9 we observe two cases of trisomy 21 (Down syndrome) in trophoblast samples from the cervix of the pregnant woman. In image A we see an affected boy with one X chromosome (white arrow), one Y chromosome (blue dashed arrow) and three chromosome 21s (blue arrow). In picture B we see a corresponding little girl (two X chromosomes).

In general, targeted FISH based on morphology [68,69] and/or immunophenotyping [22,45,70-72], should be the method of choice in all cases where the number of cells examined is very low, e.g., detection of minimal residual disease [68,70,73], detection of circulating tumor cells [22,72], and chimerism after hematopoietic progenitor cell transplantation, or when separation by any other method is difficult [26,45,69,71].

In Figure 10 we see the localization of cancer polyploid cells in peripheral blood of patients with bowel cancer (A) and ovarian cancer (B).

Figure 11 shows a comparison of cells from patient sputum and the corresponding FISH analysis. In section (a) a morphologically normal cell is shown, but in analysis (d) it appears to be missing chromosome 3. In the other sections, the morphology is combined with pathological FISH signals, polyploidy or ellipsis.

Figure 12 shows an example of combined immunophenotyping and FISH analysis. In panel A, CD10-positive lymphoblastic cells are seen, but of these, by FISH, only two have the ETV6/RUNX1 mutation (arrows, B/1, B/3). Conversely, in sections B/2 and B/4 we observe cells that are immunophenotypically positive for CD10, i.e., they are germline but do not contain the mutation in question. Panels C and D show bladder cancer cells by both immunophenotype staining and their numerical perturbations by multicolor FISH.

To detect rare events, e.g., minimal residual disease in patients undergoing cancer therapy, various techniques based on polymerase chain reaction and flow cytometry are the most preferred methods. However, molecular genetics techniques are not cell-based methods and thus provide only an indirect estimate of the true tumor burden. In addition, a pathological phenotype different from that of normal cells is not always present, or may be lost during clonal evolution making the interpretation of flow cytometry results difficult. These

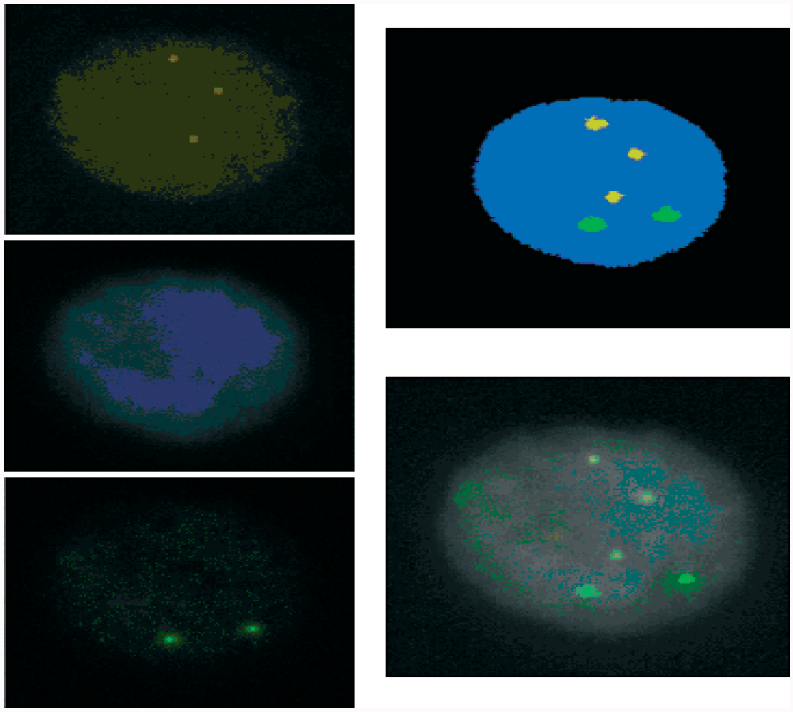


Figure 8: Detection of trisomy 21 (Down syndrome).

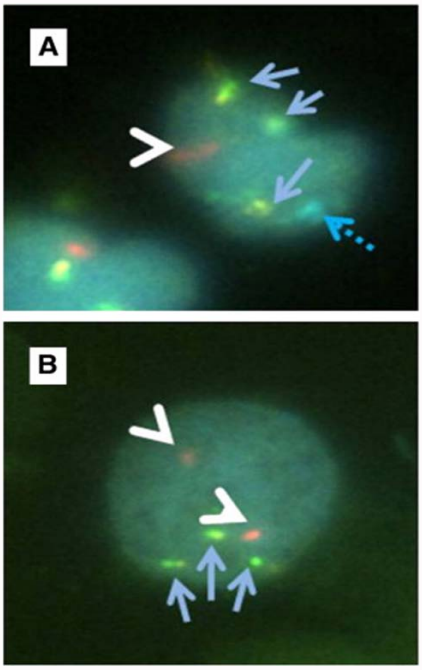


Figure 9: Two cases of trisomy 21 (Down syndrome) in trophoblast samples from the cervix of the pregnant woman.

topics demonstrate the suitability of cell-based methods in the combined collection of morphological, phenotypic and genotypic traits at the single cell level. Of major importance are also some specific applications of automated analysis including the quantification of telomere length revealing their abnormal structure [18,23,74], and the control of the exact location of a certain chromosomal region [20]. These tests, however, are not yet part of clinical practice, but in fact demonstrate the importance and "viability" of automated FISH analysis.

In a different but also interesting field of science, that of environmental microbiology, the automatic analysis of FISH images helps in the direct detection of cells from environmental materials such as soil, chemicals and natural fertilizers with the ultimate goal of identifying microbes [75,76].

Discussion

In situ hybridization was developed in the late 1960s to early 1970s, and fluorescent labeling appeared a decade later. Due to the ever-increasing efficiency of marker probes, the popularity of FISH rapidly began to increase in the early 90s. It was not long before cytogeneticists realized that the evaluation and even the manipulation of the data is very laborious, requiring expertise and high resistance, especially, in cases of analyzing a large number of cell nuclei. Therefore, it came as no surprise that experts almost immediately began to consider various possibilities to automate the process.

Various successful attempts have been made, and with the exception of some early technical studies the two primary goals have always been:

(i) Reach or even exceed expert assessment accuracy, and

(ii) Create a fast and efficient medium, requiring as little supervision as possible [77].

Regarding the first goal, even if high-accuracy systems have been created based on the relevant literature, it is clear that the level of this characteristic will go even higher, getting closer to human interpretation. The reason for this is that the evaluation of the FISH signal requires the ability to accurately identify and interpret the image produced. The systems already developed approach human interpretation with very good results. The studies evaluating automated FISH image analysis systems and comparison with

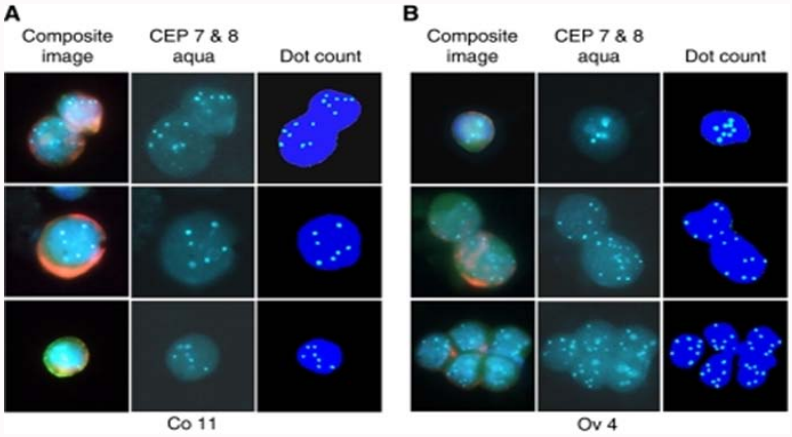


Figure 10: The localization of cancer polyploid cells in peripheral blood of patients with bowel cancer (image A) and ovarian cancer (image B).

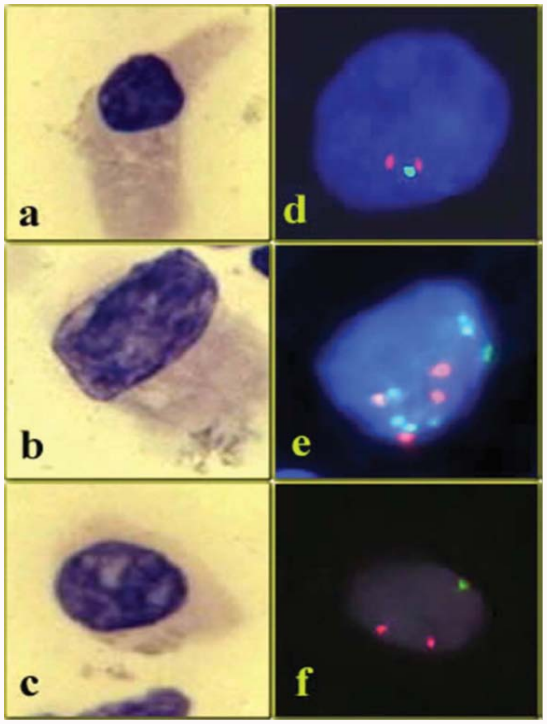


Figure 11: Correlation between morphology and FISH analysis.

the non-automated method, listed in the corresponding chapter, demonstrated perfect agreement in several cases.

FISH signals are not uniform, and the types of probes, target regions, choice of fluorochrome, sample type and handling are factors that affect the morphological variability of the signal and may lead to various types of noise that mimic the signal that could disturb the interpretation of the generated image [77]. The varied and complex definitions of positive cell tests, together with atypical and abnormal signals make their interpretation even more difficult. That's probably why in most articles it is very difficult to understand whether the method used is automatic or semi-automatic, confusing even the concepts themselves. Since the definitions are common to both non-automatic and automatic analysis, the aforementioned interpretation tactics are also used to train automatic analysis systems. Consequently, the same problems faced by the expert during the evaluation for successful identification of the biological phenomenon will be faced by the automatic system, leading to the same percentage of errors.

On the other hand, regarding the second aforementioned goal, human labor would obviously not be able to compete with the speed, consistency and endurance of these systems. In the case of automatic analysis, fatigue and speed do not alter the results and as long as the preparation conditions do not change dramatically, consistent and objective results are obtained. Although manning an automated microscope workstation is expensive [78], automated analysis has advantages:

- Automatic analysis allows the investigation of a large number of cells without increasing the required time [6]
- The reliable detection of positive cells presented in a very low percentage could become available through the examination of a high number of nuclei [53]
- Objective criteria make the analysis independent of the skills and bias of the evaluator [79],
- Automation eliminates exploratory variability and produces higher statistical reliability than non-automated analysis even when examining the same number of cells [79]
- The fields and slides of the digital image allow the repetition of morphometric measurements without attenuation of the FISH signals,
- Each study object is stored in the collection created, providing an excellent documentation of the analysis [79],
- Due to the relative collection of the morphometric parameters the characteristics of the cores can be analyzed in any order and combination. Therefore, these systems not only analyze FISH signals, but also enable complex cytometric investigations,
- The coordinates of each object are stored allowing fast and accurate repositioning, which is a prerequisite for the continuous investigation of morphology, phenotype and genotype at the single cell level [70]
- The automatic analysis facilitates the objective assessment of histological samples which is often difficult, due to the presence of incomplete, tangential and overlapping cores,
- Cell-based detection of minimal residual disease, circulating tumor cells and clonal progression is possible.

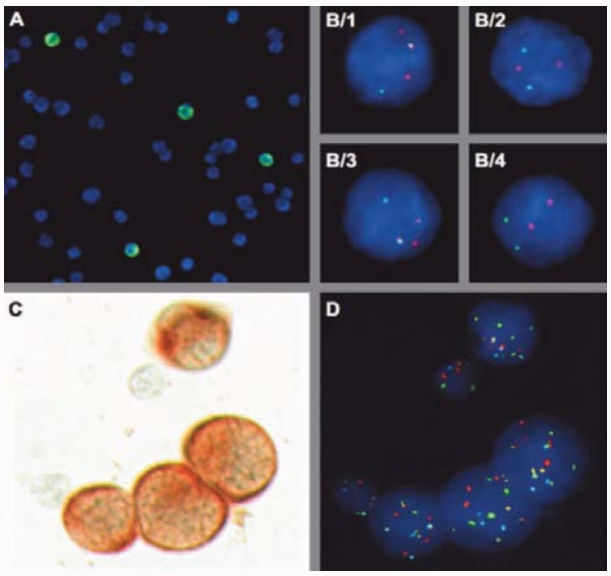


Figure 12: Immunophenotyping and FISH.

Based on the above it is clear that the use of automated FISH processing and analysis systems is very important. Nevertheless, based on the relevant literature, we are led to the conclusion that that there is a lack of experimental protocols that would lead to objective and sufficient results. Usually, the definitions used in the presentation of the experiments and results are not fully harmonized with the international nomenclature of the scientific subject. Harmonization with the international nomenclature will lead to the development of a protocol for the design and implementation of automatic processing and analysis systems. This protocol will bring closer the time of total automatization of FISH techniques.

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