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The Feulgen reaction: from pink-magenta to rainbow fluorescent at the Maffo Vialli's School of Histochemistry

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ABSTRACT

For over a century, Palazzo Botta (Botta Palace) has housed the University of Pavia's Biomedical Institutes. Illustrious scientists have conducted research and taught at this Palace, making significant contributions to the advancement of natural, biological, and medical science. Among them, Camillo Golgi received the Nobel Prize for discovering the so-called "black reaction." Following Golgi, the Palace continued to be a hub for the development of methodologies and reactions aimed at detecting and quantifying biological components. Maffo Vialli (in the Golgi stream) was the first to establish a Histochemistry Research Group, which began in the naturalistic field and later expanded to the biomedical area. Among the many histochemical studies initiated in the Palace, the Feulgen reaction undoubtedly played a significant role. This reaction, developed by R. Feulgen and H. Rossenbeck in 1924, had significant international implications: numerous researchers then contributed to define its fine chemical details, which remained the subject of study for years, resulting in a massive international scientific literature. The Pavia School of Histochemistry also contributed to the evolution and application of this method, which has become a true benchmark in quantitative histochemistry. Giovanni Prenna and the CNR Centre for Histochemistry contributed, as they were already focused on fluorescence cytochemistry. The Pavia researchers made significant contributions to the development of methodology and, in particular, instrumentation; the evolution of the latter resulted in the emergence of flow cytometry and an ever-increasing family of fluorescent probes, which somewhat overshadowed the Feulgen reaction for DNA quantification. The advent of monoclonal antibodies then allowed the final explosion of flow cytometry in clinical application, almost making young neophytes forget that its roots date back to Feulgen.

Key words: Feulgen reaction, quantitative cytochemistry, fluorescence microscopy, flow cytometry.

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Introduction

The first histochemical reactions were undoubtedly discovered in the nineteenth century, but their detailed chemistry, and thus the emergence of a true scientific discipline of histochemistry, occurred in the twentieth century. In this sense, Botta Palace (Figure 1, formerly the first location of the University of Pavia's Biological Institutes) was one of the sites where this science grew and developed, first with Camillo Golgi and then with Maffo Vialli (Figure 2), the pioneer and tireless animator of the Pavia School of Histochemistry. Prof. Vialli's long and successful career resulted in the formation of a highly motivated research group that was particularly active on three major histochemistry-related topics between 1950 and 1970: animal anatomy and histology, the proposal of new quantitative methods for DNA determination, and the development of instrumentation.

Prof. Vialli, a great nature lover and talented comparative anatomy teacher, devoted his early research to morphological functional anatomy in vertebrates, with a focus on amphibians and mammals. He quickly learned how to look beyond simple histological findings in search of structure-function relationships that persist in animal models and even some plants. His exceptional abilities as a teacher and researcher, combined with his warm personality toward his students and coworkers, enabled him to approach people from various backgrounds in biology and medicine. In fact, he became a reference point for many young physicians who, under his guidance, conducted medical research on a variety of subjects. He established a National School of Histochemistry with his colleagues, and in 1954 also founded the *Rivista di Istochimica normale e patologica* (now *European Journal of Histochemistry*). Prof. Vialli made significant contributions to the disciplinary autonomy of histochemistry, which until the middle of the twentieth century was only viewed as an investigative support for morphological investigations.

Among the School's first distinguished medical students, Vittorio Erspamer stands out: Vialli's early knowledge of the "enterochromaffin cell system"[1] enabled him to delve deeply into its molecular components, bringing him close to receiving the Nobel Prize. That substance, which Vialli had previously identified as enteramine in the gastrointestinal tracts of fish, amphibians, and mammals, became the most well-known 5-hydroxytryptamine, with Erspamer defining it as the progenitor of a family of neurotransmitters.[2] Looking back, in Botta Palace with Vialli and Erspamer, what had happened with Camillo Golgi and Santiago Ramón y Cajal was repeated - more or less half a century later.[3] The study on the enterochromaffin cell system is undoubtedly one of the three research lines of the Vialli School of Histochemistry, which has been active for at least thirty years.

The other two research lines of the School mentioned above, are undoubtedly more distinguished by their histochemical content. The first focuses on the development of methodology for the quantitative determination of nucleic acids, while the second focuses on the development of instrumentation for obtaining quantitative information from histochemical reactions. These were the paths Prof. Vialli chose to direct the research of his closest students and collaborators, who, along with him, defined the most significant findings of the second half of his research journey. He was always most faithful to his animal models, even as the evolution of various clinical orientations gradually shifted the focus of scientific interest away from biology and toward histochemistry applications in medicine.

The Feulgen reaction

Early discoveries that DNA was the foundation of cell replication and that abnormalities in DNA content were closely linked to neoplastic transformation drew most researchers' attention to this nuclear component. The reaction developed by Robert Feulgen in 1924,[4-6] was the turning point of this research, and a flood of literature after him demonstrates the interest that was devoted to validating the stoichiometric conditions of this method.[7-9] The application potential of this reaction is based on the concept of "quantitative evaluation",[10] which distinguishes between normal (diploid) and abnormal (aneuploid) DNA content. This fine differentiation would later serve as the foundation for much clinical research into normal and neoplastic cell populations

The Feulgen reaction became a daily occurrence in the Palace during the 1960s and 1970s, and all young researchers/students from that era have vivid memories of it. The pungent odor of sulfur dioxide, as well as the prominent fuchsia/magenta stains that invariably adorned white lab coats, are distant memories. Personal

Figure 1. The Botta Palace (1700) was originally the private residence of the family Botta-Adorno, and from 1890 to 2010, the location of the Biological Institutes of Pavia University (as it appears on top of the main entrance of the building: center panel). It currently houses the Golgi Museum (right panel) and the University's Kosmos Museum of Natural History.

Figure 2. Professor Maffo Vialli and some of his historical collaborators attended a convivial event in the Comparative Anatomy Institute's library, in the 1970s.

protective equipment (but also fume hoods) at the time were far from what they are today, and many of the reagents used in histochemistry (beginning with formalin) were "unpleasant" to those who had to handle them. In this sense, the mythical Feulgen reaction left traces in many of the Palace's rooms and laboratories!

The advent of "quantitation"

Initially, the Feulgen reaction was used primarily to highlight cell nuclei in tissues, often in conjunction with other colored markers (*e.g.*, for proteins, lipids, saccharides, etc.) for morphological observations using conventional bright-field microscopy. Prof. Vialli and his School made a significant contribution by pioneering the concept of quantitative histochemistry on a global scale.[11-13] He worked with some collaborators (Cesare Casella, Sergio Perugini, Luigi Zanotti) to create the first combined instruments for observation and measurement (Figure 3), that he dubbed histophotometers.[14-16] He thus marked the evolution of histochemistry from a simple support for morphological observations to a research tool for quantitative evaluations of cellular components. Many factors contributed to this, which he was able to direct in his favor, most notably the rapid technological development of those years and the availability of instrumental goods of military origin (post-war), which he was able to obtain and which he and others worked tirelessly to transform or adapt to set up measuring instruments.[17-19]

From the first pioneering (and approximate) histophotometers, the evolution led to cytophotometry, specifically nucleus photometry, which was primarily based on the Feulgen reaction. Cytophotometry was based on determining the "density" of staining expressed by the Feulgen reaction, *i.e.*, the classic magenta color that characterizes the cell nuclei at the end of the staining procedure. Through a complex analytical approach known as "microphotometry," the beam of monochromatic (green) light first passed through the nucleus and then through a "white field," and the density and thus the content were calculated from the ratio of the two readings. Many of us, young (at the time) researchers, spent hours at the microphotometer gathering data on hundreds of cells for methodological or for applied research purposes. Vialli's rudimentary histophotometers had evolved into commercial instrumentation, such as the historic Leitz MPV1 microphotometer, which is now a museum object (Figure 4).

The first commercial instrumentation only offered the possibility of densitometric measurements to read a single field, defined within the nucleus, by a special diaphragm and thus of a similar reading "outside". Single-reading cytophotometry, which was gaining popularity, quickly revealed one of its most significant limitations, which does not exist in the field of "cuvette" photometry, namely the so-called distributional error.[20-22] This is due to the fact that, unlike a sample in a cuvette, the cell (or rather the nucleus in the case of DNA analysis), exhibits a significant heterogeneity in the distribution of its internal components. In fact, it is known that the chromatin in the nucleus is heterogeneously distributed, and that this heterogeneity varies according to the cellular phenotype. Furthermore, the cell's functional state influences chromatin condensation in different parts or throughout the nucleus. Chromatin decondensation characterizes specific functional conditions, such as cell proliferation. To overcome the distributional error, absorption measurement techniques were developed based on a system of integrated readings across the entire surface of the sample.[23,24]

Various technologies were proposed, primarily distinguished by two different scanning systems of the object under examination (cells on glass slides). The first system involved moving the microscope stage, which houses the slide, in relation to the beam of light sent to the specimen *via* a small diaphragm. A second system, which discovered greater diffusion, enabled the movement of the light beam through various and sophisticated optical-mechanical devices. Among these are those that used a perforated disk (which rotated between the lamp and the objective) and others that used oscillating mirrors; both systems are still used in advanced microscopies such as confocal microscopy.

Maffo Vialli (and later his pupils Giuseppe Gerzeli, Maria Gabriella Manfredi Romanini and Giovanni Prenna) always prioritized quantitative histochemistry research and thus invested heavily in analytical instrumentation. By the 1960s/70s, in addition to the "classic" Leitz microphotometers, two scanning microphotometers had been acquired: the "old" Barr and Stroud, with optical scanning made by a perforated rotating disk, and the more modern Vickers M85 with optical scanning by an oscillating mirror. The use of these instruments was quite demanding, and the amount of data collected was frequently limited by the time required to perform the analyses, which were always carried out manually, cell by cell.

Fluorescence beats absorption

In the 1950s and 1960s, thanks to Vialli's efforts, the School of Histochemistry was further consolidated with the establishment, in 1957, of the "Centre for Histochemistry", a new national research body established and funded by the National Research Council; Vialli initially directed the Centre. Since its inception, the Centre maintained a clear methodological/instrumental orientation focused on quantitative cytochemistry, particularly fluorometry.[25]

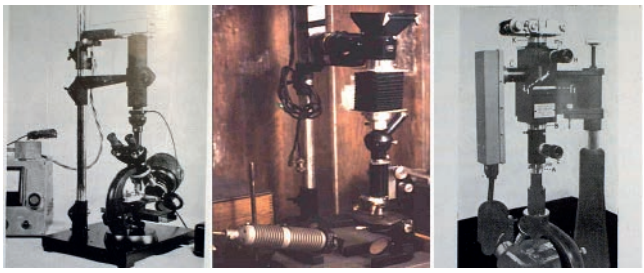

Figure 3. Different models of home-made histophotometers by Vialli's group.

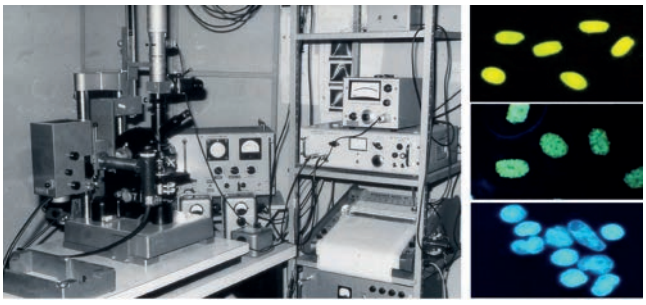

Figure 4. The first commercial Leitz MPV1 cytophotometer and (right) micrographs of frog erithrocyte nuclei that were Feulgen-labelled with different Shiff-type reagents.

In the following years, Vialli handed over control of the Centre to Giovanni Prenna, who, with some collaborators, initiated a research program aimed at developing new, particularly efficient Schiff reagents for use as fluorescent probes for the Feulgen reaction. Many international groups and researchers in the Palace have been working on this reaction and its applications for quantitative DNA determination: among them, Vialli's collaborators, Gerzeli and Manfredi Romanini were working on various biological problems on animal models, while visiting clinicians began to study tumor proliferation and biological characterization using quantitative DNA detection.

Vialli's histophotometry, which was initially semi-quantitative, began to refine and become more precise, eventually evolving into cytophotometry for the quantitative determination of cell components, with DNA content remaining the most studied. Higher precision had become critical for biological species characterization as well as studying proliferation and DNA content abnormalities in human neoplasms. This had been the motivation behind improving methods and instruments, eventually transforming cytophotometry into cytofluorometry.[26,27]

Meanwhile, technological advancements in the 1960s resulted in the widespread use of fluorescence microscopy, which became an important tool for quantitative cyto-histological studies. Parallel to the spread of fluorescence microscopes, fluorescence methodology began to evolve as a result of the contributions of many laboratories: classical cytochemical reactions for demonstrating cellular components were transformed into fluorescence methods. Fluorescence determination had already replaced absorption techniques in biochemical laboratories due to the advantages of sensitivity and precision provided by fluorescence. Since the fluorescence measurement is a direct approach that measures the fluorescence intensity of the fluorochrome associated to the structure to be determined, the measurement procedure is simpler and, most importantly, eliminates the distributional error that affects the microdensitometric measurements.

The Feulgen reaction must be regarded as a watershed moment in both the history of DNA quantitation and the development of DNA fluorescent cytochemistry. Many researchers around the world have focused their studies on the evolution of Feulgen reaction as a result of the development of new fluorescent probes.[28-31] The original Shiff-reagent used to bind the carbonylic groups produced by acid hydrolysis is suitable for absorption microphotometry due to its magenta color, but it is not suitable for microfluorometry due to its poor fluorescent nature.

In the early 1970s, Johan Sebastiaan Ploem[32] established a fluorescence lighting system (which is still commonly referred to by his name), revolutionizing fluorescence microscopy and all cytofluorometry instrumentation. The new device (the epilluminator or incident light illuminator) can illuminate the sample with great efficiency while also minimizing the excitation light component that can reach the observer's eye (or the photomultiplier). The end result is a higher-quality image, even with weak fluorescence, and thus increased sensitivity and measurement accuracy.

Fluorescence cytochemistry evolved alongside fluorescence microscopy. Microspectrofluorometers were developed in response to the need to characterize fluorescent substances chemically and physically and to assess their suitability for use in quantitative applications.[26] These instruments are based on fluorescence microscopy and can analyze the spectral distribution of the emitted fluorescence (evaluation of the emission spectrum); they also allow variable excitation lights to continuously explore the light spectrum in all ultraviolet and visible frequencies. In this way, the excitation spectrum of the fluorochrome linked to the biological structure is evaluated. The microspectrofluorometry therefore can reveal the excitation and emission characteristics of the fluorochrome under examination, defining the optimal excitation conditions and observation strategies.

From single color to fluorescent rainbow

As mentioned above, the original Feulgen reaction, which was largely used in quantitative absorption methods, proved to be unsuitable for microfluorometry. This was due to the original dye's poor fluorescence emission (pararosaniline or basic fuchsin), which, in addition to a low emission efficiency, exhibited other spectral characteristics that may alter the linearity of the fluorescence to dye concentration relationship.

Giovanni Prenna began a research project at the CNR Centre's laboratories with the goal of developing new reagents to replace

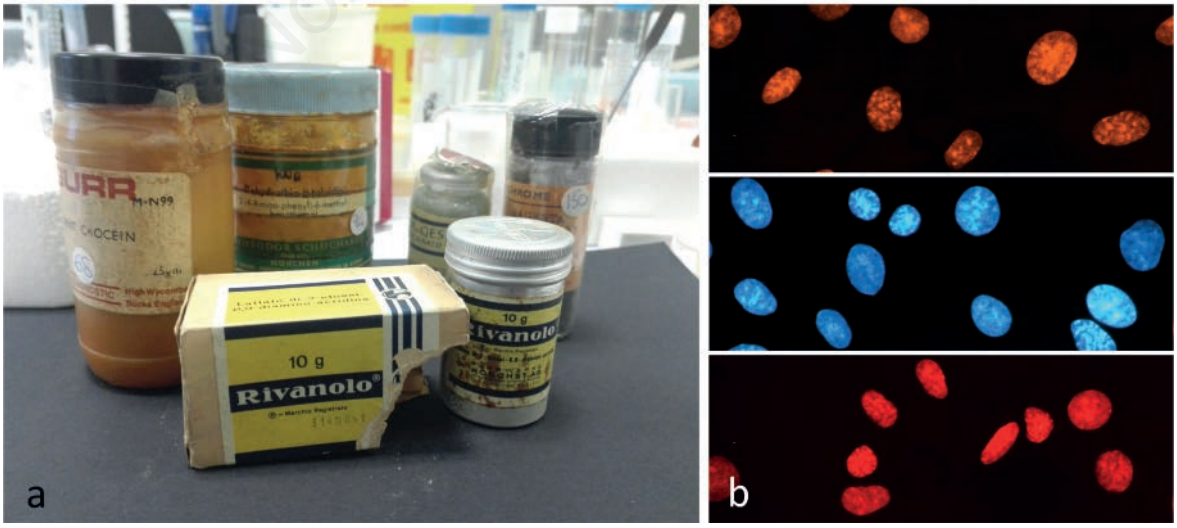

Figure 5. Some of the chemical drugs tested by Giovanni Prenna as possible fluorescent Shiff-type reagents and examples of Feulgen-stained HeLa cell nuclei.

pararosaniline in the Feulgen reaction.[33-36] The goal was to test new fluorochromes with higher efficiency and spectrofluorometric properties that were more suitable for the analytical setting of microfluorometry. As previously stated, the classical pararosaniline Feulgen was characterized by low fluorescence emission and spectral disadvantaged in the emission in the deep red; at this wavelength, the photomultipliers of early microfluorometers (*e.g.*, the 1P28 phototubes) were insensitive, and only the evolution of sensors and the use of the modern S20 phototubes allow efficient measurements even in this spectral range. As a result, fluorochromes with the most favorable spectral regions were sought, and Prenna experimented with several molecules (initially medical drugs and their derivatives: Figure 5) capable of transforming into "Shiff-type-reagents" with brilliant emissions in the blue/yellow range of the visible spectrum. A second significant advantage of these new fluorochromes was their ability to be excited in the highly favorable ultraviolet spectral region. Mercury lamps, which are still used in fluorescence microscopy and microfluorometry today, emit a significant amount of energy precisely in the ultraviolet, blue and green bands. The use of some of these Shiff-type probes provided important advantages in the quantitative determination of DNA, particularly in terms of sensitivity and precision. The experimental application of these fluorochromes, combined with the availability of a new set of fluorescent markers, paved the way to "Rainbow-microfluorometry", which laid the groundwork for multiparametric analyses (Figure 6).

The need to investigate the spectral (excitation and emission) properties of new molecules in depth and *in situ* prompted the Center's research into microspectrofluorometry.[26] In fact, Prenna was a pioneer in this field also, having oversaw the installation at the Centre of an early Leitz instrument dedicated to such research.[37] For many years, the Palace was also the site of experimentation and interaction between experts from this Company and CNR researchers working on the development of advanced microfluorometric instrumentation.

The instrumental evolutions

In the same years, Giovanni Prenna established an important collaboration with the "Quantum Electronics Group of the CNR," at the Politecnico of Milan, allowing for a brilliant and fruitful new parenthesis of technological research, aimed at the development and construction of new instrumentation for microfluorometry and spectrofluorometric analysis with high sensitivity and precision. This collaboration resulted in some instrumental prototypes that incorporate all of the latest technologies. Sergio Cova introduced an absolutely innovative measurement system based on single photon counting and fluorescence signal acquisition with digital techniques, which will be used for both quantitative cytofluorometry instrumentation and spectrofluorometric analysis. Parallel to the development of analytical systems for detecting and measuring fluorescence signals, a component of research into new excitation sources emerged. Again, a collaboration with the Politecnico of Milan, involving electronics and physics-optics experts, resulted in notable experimental contributions to the introduction of laser sources in cytofluorometry.[25,26] The results obtained from the use of power laser sources are especially important both in terms of technological development and application. The studies conducted with pulsed laser sources to obtain new fluorochrome characterization parameters, such as the decay time of the fluorescence pulse, are absolutely unique and of international relevance. Its fine and precise analysis (made possible by the innovative instrumentation developed) provides important morpho-functional information about the microenvironment in which the fluorochrome is located.

Flow cytometry, the last chance!

In the history of cytometry technology, the development of flow cytometry was a watershed moment, thousands of cells to be measured in seconds.[38-43] As early as the 1960s, a group of

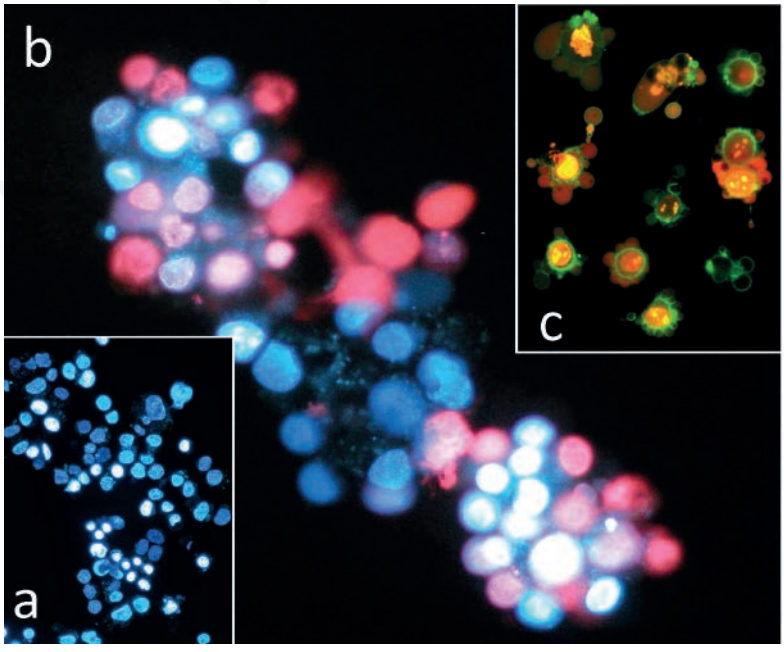

Figure 6. Multicolor fluorescent examples of rainbow labeling. DHD cells: **a**) supravital single labelling with Hoechst 33342; **b**) supravital dual-labelling with Hoechst 33342 and propidium iodide; **c**) apoptotic HeLa cells after supravital dual-labelling with FITC-conjugated Annexin V and propidium iodide.

researchers in the United States developed the first automated cell counting systems using two different physical principles: the first based on impedance measurement (Coulter's principle), and the second devised by Herzenberg based on optical analysis (light scatter).[26] The first family of instruments evolved into the so-called cell counters, which are still widely used in all analytical laboratories for automated counting, mainly of the various cell subpopulations in the blood (or other body fluid). The second group of instruments evolved in terms of quantitative analysis of cellular components to the present flow cytometers, which became commercially available worldwide, a few years later. These are primarily distinguished by the principle of the "hydrodynamic focusing" of the cells at the point of analysis, which ensures a precise, constant and individual illumination of the cells, as well as the collection of qualitative-quantitative data resulting from their interaction with the excitation light beam. Two instrumental lines can be identified that derive from two different constructive principles, which clearly have specific advantages and limitations (the subject of recurring scientific and commercial diatribes). On the one hand, a group in the United States[38] created a system based on an innovative (at the time) laser lighting concept that was highly adaptable to this application. Another group in Germany[43] created a lamp-based flow analysis system based on a fluorescence microscope in epillumination, replacing the slide with a special flat flow chamber to allow the cells to flow into the analysis point.

In the early days of flow cytometry, the focus was on DNA and thus the Feulgen reaction,[44] and the Vialli School made an important contribution in this regard. In the 1970s, Giovanni Prenna was already in contact with researchers (both in Germany and in the United States) for his research on fluorescent Shiff-type reagents, and at a conference in Seattle, he met Louis Kamentsky (the first flow cytometry manufacturer in the United States), with whom he maintained close contacts until he obtained the installation of an early Biophysics 4800A instrument at the Pavia Centre. Prenna's group began experimenting on this instrument with the first applications of acriflavin as a substitute reagent for pararosaniline for DNA determination. A second flow cytometer (ICP Partec PAS) was installed in Pavia as a result of a profitable collaboration with Wolfgang Göhde's group. As previously stated, this second instrument used a conventional excitation source, a mercury lamp that provided a particularly efficient illumination in the ultraviolet region. Since the new fluorochromes tested by Prenna were all best excitable in this band, the collaboration with Göhde became particularly fruitful. In particular, a fluorochrome named BBT was successfully tested for its application on this instrument.[45]

Multiparametric analysis of other DNA-related cellular components was gradually becoming more common in many areas of the biomedical research. Already in the earliest applications of flow cytometry, devoted to studying cell kinetic features, early attempts were made to measure other significant parameters in cell proliferation, such as the S-phase fraction after labeling with the thymidine substitute, bromodeoxyuridine (BrdU; Figure 7). Despite its limitations due to its nucleic acid binding mechanism, acridine orange[46,47] was the first dye used for multiparametric analyses aimed at correlating DNA and RNA contents in single cells. The concurrent use of the fluorescein derivative, fluorescein isothiocyanate (FITC), for protein tagging and the development of new DNA labelling methodologies marked the beginning of true multiparametric analysis.[48-50] The propidium iodide-FITC pair proved to be ideal for applications with cytofluorometers that use lasers as excitation source: light at 488 nm excites both fluorochromes, resulting in easily measurable fluorescence bands. In addition to the propidium iodide-FITC pair, another combination of DAPI-SR101 fluorochromes was proposed for analysis with instruments equipped with a mercury lamp as excitation source.[43] Many of the methodological approaches aimed at improving flow DNA analysis with the goal of achieving increasingly precise results. The CNR Centre in Pavia quickly became the go-to place for the first cytometric applications in clinical settings.[51-56] Flow DNA analysis became essential in the 1980s' because it quickly

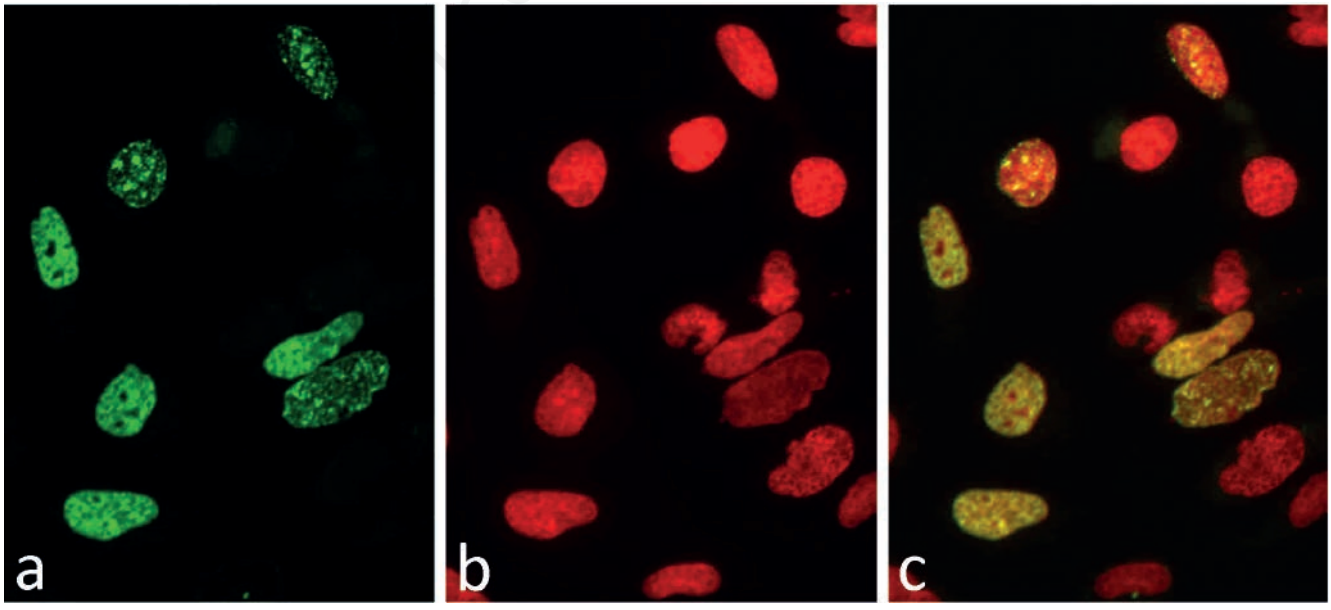

Figure 7. The advent of monoclonal antibodies. **a**) BrdU-labeled HeLa cells revealed by a FITC-conjugated anti-BrdU antibody, and **b**) counterstained with propidium iodide; **c**) merged image..

and easily provided important information about potential DNA content abnormalities (known as cytometric aneuploidies) and cell proliferation profiles. The CNR Centre helped to establish the Italian Society of Cytometry (formerly, the Italian Group of Cytometry, GIC)[57] and, through the Pavia School of Histochemistry, continued to train a large number of young scientists interested in this new technology and analytical approach.[58-61]

The enormous analytical potential provided by multiparametric methodology (including monoclonal antibodies) has rendered flow cytometry an indispensable tool, particularly in the clinical setting.[62] Although flow cytometry instrumentation is now widely used in a variety of applications ranging from medical practice to environmental monitoring, it is interesting to note that it is no longer used for its original purpose. Certainly, only few of today's young users are aware that the Feulgen reaction was the spark that ignited cytometry.

Acknowledgments

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