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Giving bodies to ghosts: locating molecules in the very place where they exert their biological roles

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ABSTRACT

This paper reviews some of the goals of our investigations published over the years on *Rivista di Istochimica Normale e Patologica, Basic and Applied Histochemistry*, and the *European Journal of Histochemistry - EJH*. In a series of papers, we published some of the basic cytochemical features of the sperm cytodifferentiation process for the first time. This was a conceptual and practical prerequisite to the in situ quantitative evaluation of sperm DNA content. We showed that the discrepancy between the expected 1:2 ratio when comparing sperm versus somatic cell DNA content (sperm DNA content is always far low from the theoretical value) is due to DNA losses caused by the hydrochloric treatment entailed by the Feulgen reaction. The knowledge of the specific losses that occur during the various steps of the Feulgen reaction has allowed us to use it critically in Genome Size studies to highlight: - sperm aneuploidy in chromosomally derived subfertility; - the broad variability range of Mammalian genome sizes; - that termites are roaches (after decades of discussion on this topic). In addition, in a seminal paper on human oocytes, we showed (by transmission electron microscopy) a specific chromatin and cytoplasmic organization (both essential for further embryo development) linked to oocyte maturation arrest, a datum quite relevant to treating unmet therapeutic needs in human and veterinary reproduction.

Key words: genome size; Feulgen reaction; sperm; oocytes.

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Celebrating 70 years of the *European Journal of Histochemistry-EJH* publishing (founded in 1954 as *Rivista di Istochimica normale e Patologica* and later, 1980-1990, as *Basic and Applied Histochemistry*) in such a competitive field as histo-cytochemistry is exciting for several reasons. One of them, probably the most emotional, is tied to the life of the master chief of this success, Maria Gabriella Manfredi Romani (1924-2010; MGMR for all the pupils), Editor-in-chief of the journal since 1984, an appointment that she directly inherited from the journal's founder, Maffo Vialli (1897-1983).

The *Rivista di Istochimica normale e Patologica* (in 1954, it was one of the first journals devoted to histo-cytochemistry ever appeared in the world, accepting publications in Italian) was the end product (the manifesto) of many efforts of the Pavia cytochemistry school conceived by Maffo Vialli with the help of Vittorio Erspamer (1909-1999), a Nobel prize candidate for several times thanks to the discovery, with the aid of Maffo Vialli, of the 5 hydroxytryptamine (serotonin) in the skin of tropical Brasilian frogs, first and in Mammals, later). Several Vialli's pupils took over his legacy, like Giovanni Prenna (1931-1977), Giuseppe Gerzeli (1931-2018), and notably MGMR. She maintained the publishing activity of the *Rivista* while facing many obstacles and difficulties till the foundation of *Basic and Applied Histochemistry* (1980-1990) after passing the scrutiny of the Thomson Reuters' Institute for Scientific Information (ISI). This led to the foundation of the current *European Journal of Histochemistry*.

We must confess our gratitude to the beloved MGMR teaching and for involving us in her basic scientific interest, which was mainly devoted to Mammalian Genome Sizes (GS). MGMR thought that to explain any biological problem satisfactorily, the *primum movens* should be known. She always identified it with the quali-quantitative features of the genome, the DNA: notably, the topographical distribution of the several DNA fractions (qualitative) that build up the genome's size (quantitative). It was an anticipatory and brilliant vision since DNA was emerging from the foggy vision we had of the role it played in any biological phenomenon during those years.

Paradoxically, the knowledge of the DNA structure (1953) left its role "underknown" and not fully appreciated for at least two decades until the molecular biology revolution.

Till the seventies, it was uncomfortable (even though exciting) to deal in detail with DNA in any explanation of biological phenomena; DNA was an "unknown," "strange," and "unfamiliar" subject. It was clear to MGMR that the evaluation of the GS should be the necessary preliminary step to highlight the role of DNA in any biological process. One of the great successes of this perspective is retrospectively simple to say: the 2001 Venter's paper[1] on the human genome sequence quotes just two Italian papers, that of Bernardi[2] on the asymmetrically distributed GC-rich fraction of the genome (the H3 isochores) and that of MGMR on the bat GS.[3] Venter and colleagues explain the inhomogeneity of the isochores distribution based on Susumo Ohno gene duplication as the primary mechanism of vertebrate evolution.[4] "Gene duplication" leads to a "desertification" (Ohno's words) of "genes distributed in a barren desert." If so, these deserts should be dispensable; thus, there must be mammalian genomes that are smaller in size than the human genome. Venter and colleagues thus came to MGMR paper on the *Miniopterus* GS entitled: "Nuclear DNA content and morphology of the karyotype in certain palearctic Microchiroptera," stating that: "Indeed, many species of bats have genome sizes that are much smaller than that of humans; for example, *Miniopterus*, a species of Italian bat, has a genome size that is only 50% that of humans (Capanna and Manfredi Romani, 1971)".

Later on, the continuous interest of MGMR in the evolutionary role played by GS was focused mainly on Primate GS and carried out by her pupils Gianfranco De Stefano, Daniele Formenti, and Carlo Pellicciari.

At that time, quantitative DNA *in situ* evaluations by light microscopy were mainly carried out using the Feulgen reaction. MGMR knew its use had to be grounded on the detailed knowledge of the reaction's molecular bases. Joachim Wilhelm Robert Feulgen (1884-1955) developed the reaction in 1914, demonstrating its use to show chromosomes in 1924. We know today that DNA exposure to hydrochloric acid cuts off the purine bases, letting free the deoxyribose, whose terminal is transformed into an aldehydic group when exposed to an acid environment. Making use of a reagent (*i.e.*, the Schiff reagent) developed by Hugo Joseph Schiff (a German chemist naturalized Italian as Ugo Schiff, 1834-1915) able to react with aldehydes group, the end product is a magenta-red material whose quantity can be reliably evaluated by the use of cytophotometric techniques (see the paper by Giuliano Mazzini, in the present issue).[5]

MGMR insisted that we clearly state the differences between histo-cytochemical staining and a reaction: the Feulgen reaction cannot be called Feulgen staining. This highlights the intellectual *magisterium* MGMR was giving us. From the biological epistemology point of view, it is unacceptable to mistake the two words and freely interchange their use (unfortunately, something is still occurring in the textbook and many websites). Thus, we conducted a detailed analysis of what was occurring to the DNA molecule when subjected to fixation, hydrolysis, and Schiff reagent exposures, focusing on the hydrochloric acid treatment (1 hour with 5 N hydrochloric acid, at room temperature). We showed that the dehistonization[6] and the hydrochloric treatment[7] produce a significative DNA loss.

The idea of MGMR (that we still share) was that cytochemical techniques can profitably be used to show *in situ* those cell genome characteristics usually studied by molecular cell-destructive techniques: in other words the idea was, metaphorically speaking, that cell biologist (who "look at" a cell) can give a "body" (thanks to cytochemistry) to the "ghost" studied by molecular biologist (who inevitably "destroy" the cell reducing it to a mere "biological reagent"). This idea sounds in the aims of the *European Journal of Histochemistry* at its forefront page: "The histochemical approach is nowadays essentially aimed at locating molecules in the very place where they exert their biological roles".

Thus, we used *Xenopus laevis* erythrocytes (at the time, the primary experimental model used to dissect the role of the repetitive DNA) to show the high potential of cytochemical technique (*i.e.*, the Feulgen reaction) to illustrate *in situ* genome molecular treats. It is well known that nearly half of the Xenopus genome is composed of repetitive fractions.[8] We showed that the 45% of repetitive DNA in the Xenopus genome could be highlighted *in situ* thanks to the Feulgen reaction based on the coincidence of our estimate (44%, as Feulgen DNA content after dehistonization and denaturation)[9] with the known 45% of repetitive DNA in the *Xenopus* genome. This finding opened the doors to *in situ* studies of the topographical features of this genome fraction in several other cell types (not only the erythrocytes used in this study).

Considering the DNA losses occurring during the hydrochloric treatment of the Feulgen reaction, we moved to highlight another intriguing phenomenon, the derailed sperm DNA content value (assessed by the Feulgen reaction) from the expected 1:2 ratio when compared to somatic cells DNA content. The dominating idea was that the highly packed DNA would not be susceptible to hydrolysis, thus leaving part of the DNA intact. This idea was strengthened by the fact that the sperm DNA content evaluation assessed by the use of intercalating DNA dyes showed non-stoichiometric values, and this discrepancy was, and it is, well explained by the highly packed degree of the paternal genome. We

show[10] that the sperm DNA loses an accountable fraction (nearly 20% of its content) under the hydrolytic treatment, thus explaining the non-stoichiometric ratio.

Cytochemical quantitative evaluation of sperm DNA content is a widespread technique used to assess the euploidy degree of sperm population for a range of applications, including the use of sperm to carry on *in vitro* fertilization (IVF) techniques (both in human and veterinary reproductive medicine). We applied this strategy to study the fate of morphologically normal but chromosomally abnormal sperm in a mouse model of Robertsonian-derived subfertility. It was found[11] that aneuploid sperm (even though morphologically normal) are selected against during the transit through the male genital tracts (epididymis and vas deferens). The female genital tracts cannot exert any selective process against aneuploid sperm that can reach the fallopian tube despite gross genome imbalance, and then fertilize oocytes. By contrast, morphologically abnormal sperm are preferentially lost before they reach the fallopian tube, suggesting they are eliminated prezygotically.

This finding allowed us to develop the "thanks to, in spite of" hypothesis to explain the sympatric process of speciation occurring in wild house mouse population:[12] in fact, sympatric house mice populations keep them isolated thanks to male subfertility and in spite of chromosomal heterozygosity.

During several decades, we got blood samples from many Mammalian species, along with collecting trips to Venezuela and Argentina: the simple idea suggested by MGMR was that the sluggish sloth (*Bradypus tridactylus*) should have a large GS. This was the case, but all the other Xenarthra GS we measured showed a large GS.[13] So, Susumo Ohno was correct (at least inside the Mammalia class!) in the sense that the oldest phyletic lines should have larger GS than the newly arisen ones. Looking for an explanation, we inspected several GS databases, finding out the GS for 373 placental mammals, of which just one was an afrotherian: still that of the pioneering 1950's work on the aardvark *Orycteropus afer* GS (5.86 pg of DNA).

We had the lucky chance to measure the GS of six selected representatives across the Afrotheria supraordinal group (using static microphotometry on Feulgen-reacted nuclei of cultured fibroblasts). It was obvious to compare the high average GS found for this group with those we got for 372 other placental mammals: 15 Xenarthra, 112 Laurasiatheria, and 245 Euarchontoglires. Thus, we demonstrated that the mean GS value of Afrotheria (5.3±0.7 pg) is the highest reported for the extant Placentalia.[13] In addition, we were able to show that the mean GS values of the Southern Hemisphere clades, Afrotheria and Xenarthra, are larger than those of the Northern Hemisphere clades, Laurasiatheria and Euarchontoglires: that is to say that the Feulgen GS is a powerful cytotaxonomical signature for each of the four supraordinal clades.

However, this grouping (based on molecular phylogenetic analyses) radically departs from the morphologically based constructions of the past, resulting in an odd collection of animals. Our data[13] on the GS variation supports the emerging phylogeny for the extant Placentalia.[14] Obviously enough, it calls for a functional explanation for what we observed.

Coming back to MGMR's idea about the small GS of Chiroptera, we recalled her suggestion about a possible functional interpretation of GS size: it becomes evident that the larger genomes in the Southern Hemisphere clades, especially Afrotheria, can be explained by adaptive functional bases. We stressed that numerous data (both on small taxonomic groups and across a whole class) show that the body mass-corrected metabolic rate of homeotherms decreases with latitude (*i.e.*, there is a positive association). The general reason is straightforward: There is no need for additional body warming, which is necessary at higher latitudes. At the same time, there is a negative correlation between body mass-corrected metabolic rate and GS: these two phenomena provide a functional explanation for the GS variation.[15]

We applied this same strategy of enlarging the GS database of a specific animal group as much as possible to benefit its phylogenomics to the long-debated termite/roach systematic question.[16, 17]

Having already used all the molecular biological tools to solve it, the debate derailed and stopped with opposing views favoring one option. When the cockroach house facility at the University of Pavia was closed, we got sperm samples for several roach species; thus, it was apparent to carry out their GS Feulgen evaluations. Using GSs as a cyto-taxonomical tool to clarify evolutionary dynamics and favor systematic groupings, we highlighted the occurrence of a polyploidization phenomenon doubling a basic GS of 0.58 pg of some termite families (superfamily Blattoidea, epifamily Termitoidae) up to the maximum GS value of 3.24 for the Blaberidae family within the order Blattodea (super-order Dictyoptera). By doing so, we confirmed that termites are roaches.[18]

The *European Journal of Histochemistry* published a detailed transmission electron microscopy study of sorted human antral oocytes[19] for the first time. IVF techniques are quite successful in providing fertility to human couples (and many other mammal species). However, many oocytes rescued after hormonal hyperstimulation fail to develop after fertilization. In a seminal paper on human oocytes, we investigate the causes behind oocyte maturation arrest by transmission electron microscopy. This was the first time that sorted human antral oocytes have been studied to seek cytological markers essential to sustain their perspective embryo development: we showed, like in the mouse model, the absence of cytoplasmic lattices and the abundance of lipid droplets in the cytoplasm of those oocytes that fail to develop behind the 2-4 cells stage. This datum and the heterochromatin distribution allowed the development of an Artificial Intelligence deep neural network model to identify the best viable immature unstained oocytes: prospectively, this opportunity will successfully increase their availability for *in vitro* maturation and IVF for the benefit of women's health.[20]

MGMR would be pleased to read the achievements of the Pavia cytochemical school she founded.

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