Ciliate research: From myth to trendsetting science

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Abstract
This special issue of the Journal of Eukaryotic Microbiology (JEM) summarizes achievements obtained by generations of researchers with ciliates in widely different disciplines. In fact, ciliates range among the first cells seen under the microscope centuries ago. Their beauty made them an object of scientia amabilis, and their manifold reactions made them attractive for college experiments and finally challenged causal analyses at the cellular level. Some of this work was honored by a Nobel Prize. Some observations yielded a baseline for additional novel discoveries, occasionally facilitated by specific properties of some ciliates. This also offers some advantages in the exploration of closely related parasites (malaria). Articles contributed here by colleagues from all over the world encompass a broad spectrum of ciliate life, from genetics to evolution, from molecular cell biology to ecology, from intercellular signaling to epigenetics, etc. This introductory chapter, largely based on my personal perception, aims at integrating work presented in this special issue of JEM into a broader historical context up to current research.

KEYWORDS
cellularization hypothesis, ecology, epigenetics, evolution, generatio spontanea, methodical developments, molecular biology

Headline news for Paramecium in the New York Times

The New York Times could not resist electing beautiful Paramecium as a “model” for its capacity of “sex without progeny” (Judson, 2010), based on the work by Piero Luporini summarized in this issue of JEM; he serves as a reporter of all “affaires amoureuses,” for example, cell–cell interactions of ciliates during conjugation (as specified below)—a century-old microscopic observation. On a molecular scale, it is based on a key-lock type interaction. Ciliates use extracellular messenger molecules, gamones (pheromones for sexual attraction). Pheromones also occur in metazoans up to humans (Johansson & Jones, 2007). Sperm capacitation during
fertilization may be a distant equivalent of gamones in ciliates (though throughout the animal kingdom perception of specific smell predominates). Ciliates also anticipate many other functional aspects.

Defining ciliate species

It is important to clearly define species before delving, for example, into evolution or ecology. Ciliates are structurally and functionally rather heterogenic, as they also appear from an evolutionary point of view. Defining ciliate species—beyond genetical data—largely depends on surface patterning including the arrangement of cortical filaments. Within the broader scope of epigenetics, these aspects are discussed here by Eric Cole and Jacek Gaertig, whereas Adam Soh and Chad Pearson envisage the regular arrangement of ciliation and the underlying filament system. Macronuclear morphology is another criterion for species identification; some ciliates are multinucleate (e.g., see Yan et al., 2017)—a characteristic outlined in this issue by Ragib Ahsan, Wumei Blanche, and Laura Katz.

EXAMPLES OF WORK ANTICIPATING LATER RESULTS WITH METAZOANS

Easy sterile mass culturing allowed for biochemical work with *Tetrahymena* that resulted in the identification of ribozymes and of telomerases (below) whose understanding became important for chromosome replication, aging, and other phenomena. To give just one more example: From the 1960s on, the mere size of a *Paramecium* cell made it easily accessible to electrophysiology (Eckert & Brehm, 1979; Naitoh & Eckert, 1969). Such analyses gave the starting point for the elucidation of ciliary function in metazoans. Some of this early work already described the deactivation of voltage-dependent Ca\(^{2+}\) channels (relevant for the formation of an action potential) by the very same Ca\(^{2+}\) after entry and binding to ciliary calmodulin (Brehm & Eckert, 1978). Remarkably, this effect was recognized in mammalian neurons only two decades later (Levitan, 1999). Beyond that, there are many more subtle details rendering some ciliates interesting models in cell and molecular biology, also for work anticipating research with metazoans.

Ciliates were of importance for understanding chromosome rearrangements. They still are important for deciphering epigenetic mechanisms (Chalker et al., 2013; Nowacki & Landweber, 2009), notably of transgenerational epigenetics. Seminal observations date back to the 1970s and even before (see below). In their present article, Martin Simon and associates (Franziska Drews and Jens Boenigk) and Caitlin Timmons (together with Shahed Shazib and Laura Katz) delve into this very topical field of nonchromosomal inheritance. For this branch of research, ciliates have become number one models, whereas this aspect of life and evolution is currently hotly debated with metazoans beyond nematodes. However, frequently that priority of innovative work with ciliates has hardly ever been acknowledged.

Ciliates as exceptional models for work honored by Nobel Prizes

To anticipate, the important seminal work of Elisabeth Blackburn, Carol Greider, and Cech, together with their associates, led to the discovery of telomerases and telomeres and of ribozymes, respectively, as appreciated below. These discoveries made with ciliates became of paramount medical importance including understanding of aging of organisms, including ourselves.

In the late 1970s, Blackburn and Gall (1978) discovered the significance of telomerases to maintain chromosome integrity; this work was followed up by Greider and Blackburn (1985, 1989), as summarized in a condensed review by Blackburn (1992). In 2009, Greider and Blackburn were honored by the Nobel Prize for Physiology and Medicine (Nobel lecture: [Blackburn, 2010]). Prerequisite for such work was the knowledge that *Tetrahymena*, like other ciliates, contains extensively fragmented chromosomes. Even more recently, this was the source of material to analyze the 3-dimentional structure of the telomerase holoenzyme (Jiang et al., 2013). *Oxytricha* with its even more fragmented chromosomes with over 16,000 telomes (Swart et al., 2013) would have been an even better source.
HISTORY OF CILIATE RESEARCH

The identification of ribozymes as an RNA species with enzymatic function, that is, as self-splicing RNAs, by Altman (1990) and Cech (1990, 2000) was another discovery without precedent; it resulted in the 1989 Nobel Prize in Chemistry. Ribozymes, for example, snRNAs in spliceosomes, transform precursor mRNA in mature mRNA. (Ribozymes also motivated another Nobel Prize laureate, Manfred Eigen, Göttingen, to believe in an “RNA world” early in evolution.) These examples illustrate once more that special features may predetermine some cells to serve as model systems, in agreement with a dictum of William Bateson (†1926), the early geneticist: “Treasure your exceptions”. In fact, after Bateson’s death, the thick, polytene chromosomes of Drosophila were prerequisite for the analysis of the dynamic structure chromosome by Nobel laureate Thomas H. Morgan. In this context, we may now think of a variety of exceptional situations in ciliates.

SERIOUS CRITIQUE OR BUMBLEDOM?

A few years ago, the popular and influential British cell biologist Munro stated in a “highly accessed article” about open questions left to solve in cell biology: “It is unlikely that the planet’s tax payers will be willing to pay for enough cell biologists to investigate every last intriguing invertebrate or bizarre bikont, and thus future work is likely to focus on particular key cell types, especially those found in tax payers themselves” (Munro, 2013). So bikonts came into the focus of this highly esteemed author and of some other reviewers. Why does he mention just bikonts like ciliates? My personal guess: They are too beautiful to be taken seriously. Consider that the very influential German zoologist and philosopher Ernst Haeckel has included also ciliates in his treatise “Kunstformen der Natur” (Art Forms in Nature, Haeckel, 1914). Yet, we shall see the advantages of their beautiful regular design for some problems of cell biology. Beyond that, in general terms political utilitarianism in science promotion is a bad counselor, as has been experienced in some countries during the current Corona pandemic.

To explain the term “bikont”: According to some evolutionary concepts (Cavalier-Smith, 2003), ciliates such as Paramecium and Tetrahymena represent an important branch of bikonts with some similarities to plants. In contrast, monokonts such as Dictyostelium (myxamoebae) and choanoflagellates are another branch assumed to lead to the evolution to metazoans. The evolutionary origin of

FIGURE 2  Documentation of Paramecium (number 16 and 17) in the book “Animalcula Infusoria Fluviatilia et Marina” by Müller (1786)
ciliates goes back to an estimated 0.8 to one billion years ago (Dorrell et al., 2013; Parfrey et al., 2011). For comparison, the age of the last eukaryotic common ancestor (LECA) could vaguely be traced back to most likely ~1.7 × 10⁹ years (Dacks et al., 2016). Therefore, it may appear justified to ask whether data retrieved with such models can be relevant at all for the most highly evolved organisms.

Surely, Munro would be right when he rejects “l’art pour l’art” work dealing with deliberate minor details without any further perspective. Nevertheless, one has to be careful with such judgment because small details may inadvertently become important. Thus, it is recommendable to look beyond a worm’s eye view and have in ones mind not only higher metazoans but also some of the “bizarre bikonts,” such as ciliates.

Therefore, what can ciliates tell us about evolution and other disciplines? Can we rediscover old concepts of design and function in highly evolved cells like ours? Considering Munro’s argumentation, usefulness for the tax payer would be the crucial criterion for public support of research. This would particularly hold true of parasites closely related to ciliates, such as Toxoplasma and Plasmodium species from the phylum Apicomplexa, the malaria causing agent. (As to be expected, these were shown to be much younger than their nonparasitic ancestors [Dorrell et al., 2013].) Jointly Apicomplexa and Ciliophora are designated as Alveolata.) Up to now, cell biology has not been good enough to eradicate such plagues since many aspects of cell biology remained unsolved. So, “intriguing bikonts” such as ciliates should be maintained on the screen of researchers because they allow for easy access to important pathogenic mechanisms. This includes variant surface antigens used for molecular camouflage and the capacity of Apicomplexa to penetrate host cells initiated by an exocytotic process. This process is enabled by the release of the contents of apically located extrusive organelles, the rhoptries that share many aspects in common with Paramecium’s trichocysts. About two thirds of a recent key review entitled “Unraveling the elusive rhoptry exocytotic mechanism of Apicomplexa” was dedicated to this aspect, under the heading: “Rhoptry exocytosis: Lessons from old studies in Ciliata” (Sparvoli & Lebrun, 2021)—a clear declaration of belief in the value of ciliate research. (Note that there are also important features in common to, or deviating from ciliates [Plattner et al., 2012].) Though the claim that research should not be pursued with every “last intriguing invertebrate or bizarre bikont” (Munro, 2013) is somehow understandable, it definitely must be attenuated, modulated, and even abandoned for many topics addressed throughout this issue.

Surely, Munro’s statement gives a negative connotation to work with bikonts. However, analysis of unicellular bi- and monokonts, including painstakingly detailed description of species for systematics, is valuable not only for determining the evolutionary context but also to fit these cells into terrestrial (Foißner & Berger, 2021), marine, and limnic ecological systems. They represent highly important members of the food chain and quality indicators (Fenchel, 1987; Foißner, 1999; Foißner & Hauksworth, 2009). In this JEM special issue, T. Weisse and D. Montagnes give an account of aquatic ciliates, with the emphasis on “conceptual understanding of how ciliates will respond to climate change”. Currently, this is an urgent aspect in ciliate ecology (Lu et al., 2021).

Surely, one has to know every morphological detail of “every last intriguing…bikont,” but such knowledge was a necessary prerequisite for molecular barcoding before ciliates and other protozoa could be used for more extensive ecological analyses. This was complemented by molecular barcoding on the basis of 18S rRNA. This together with fluorescent in situ hybridization is leading, as summarized, for example, by Lara and Acosta-Mercado (2012) for bioindicator species in soil. Boscaro et al. (2018) have made generally available a phylogeny-based database of small subunit rRNA for ciliates. This greatly facilitates and accelerates systematic and ecological work. Together with other protozoa, ciliates form a link in freshwater food chains (Porter et al., 1979) with their seasonal variations (Pitsch et al., 2019).

Along these lines, another branch of research is presented here by Laura Katz and her associates (Caitlin Timmons and Shahed Shazib); they trace the integration of viral (and bacterial) genes into the nucleus of ciliates and up to mammalians—a concept of high actuality. Currently, we realize how important zoonoses are. Could ciliates or any other eukaryotic microorganisms provide reservoirs for novel viral pathogens? This aspect emerges impressively in discussions in consequence of the current coronavirus 19 pandemic.

Current work on secondary endosymbiosis of bacteria, as outlined in the chapter by Masahiro Fujishima and Yuuki Kodama in this issue, can contribute to an understanding of the evolution not only of organelles of endosymbiotic origin, such as mitochondria and chloroplasts, but most recently also of the apicoplast in malaria parasites as a derivative of an endosymbiotic Rhodophycean. The apicoplast has been envisaged as a target for malaria defense, as its residual plastid DNA encodes parasite-specific enzymes for fatty acid and isoprene synthesis. Some of the aspects of symbiont homing in ciliates are currently subjects of research with potential importance. Considering the established zoonose-type interactions between free-living organisms and epidemic catastrophes, it appears unsustainable to push aside organisms of allegedly less practical importance.

## INCREASING SELF-CONFIDENCE OF RESEARCH WITH CILIATES AS MODELS FOR ALL SEASONS

As to be shown in this issue, the ciliate community has indeed many good arguments to reject the generalized
reservations of influential colleagues like S. Munro. In fact, the scientific and practical importance of ciliates ranges over many disciplines, from evolution, endosymbiosis at different levels, ecology, molecular biology, epigenetics, etc. Therefore, it is now important to show to a broader readership the wide range of topics and the real capacity of research with ciliates. Toward this goal, many colleagues with different research backgrounds have addressed widely different aspects of ciliate life. This corresponds to the intentions of the founders of the Journal of Protozoology, as formulated in the first issue: “At the business meeting in September 1953, the proposal to establish a quarterly journal was overwhelmingly approved and the title Journal of Protozoology was adopted.” Since then, this journal has maintained its multidisciplinary character, and since 2000, with its new title, Journal of Eukaryotic Microbiology (JEM), it includes other unicellular eukaryotes as well.

Beyond examples given above, there is a variety of additional innovative work. One example of ciliates as useful models is the requirement of calmodulin in establishing the docking sites of secretory organelles in *Paramecium* (Kerboeuf et al., 1993). The same was reported for mammalian cells, including chromaffin cells and neurons, though with considerable delay (Quetglas et al., 2002; Wang et al., 2014). Another example is the unexpectedly rapid exocytosis–endocytosis coupling in *Paramecium* (Plattner et al., 1992), which, in the 1990s, was without precedent. For mammalian cells, this was established in neurosecretory cells by advanced patch-clamp technology (Thomas et al., 1994). Previously, duration of exo-endocytosis coupling had been unanimously overestimated by orders of magnitude. A crucial parameter was the induction of synchronous trichocyst exocytosis in *Paramecium*. A technical prerequisite was the development of a highly efficient cryofixation method, enabled by an appropriate mixing chamber combined with spray-freezing, that is, spraying into liquid propane as a very efficient cryogen (Knoll et al., 1991), combined with freeze-etching analysis of exocytosis sites. In our hands, spray-freezing, that is, spraying into liquid propane, has been found to be most efficient in producing such surface proteins (Azzouz et al., 1995).

Inhibitors would be needed to affect the formation of such surface components in Apicomplexa, thus making them amenable to antibody response. Work with ciliates could be a starting point for screening studies and further approaches with the parasites. In *Trypanosoma*, Ko and Thompson (1992) have characterized immobilization antigens as glycosyl-phosphatidylinositol-linked proteins. Also in *Tetrahymena*, Leondaritis et al. (2011) have characterized multiple phosphatidylinositol- and phosphatidylinositol 4,5-bisphosphate-specific phospholipases C (PLC). In *Paramecium*, the PLC-type enzymes responsible for shedding glycosylphosphatidylinositol-anchored surface proteins are secreted. These forms are different from those responsible for intracellular inositol 1,4,5-trisphosphate (InsP$_3$)signaling (Staudt et al., 2016) required for locally activating Ca$^{2+}$-release via InsP$_3$-receptor type Ca$^{2+}$-release channels (InsP$_3$R-type CRCs). Thus, theoretically one could address two possible strategic sites. As discussed below, a second theoretically possible pharmacological approach to stop infection by Apicomplexa parasites could be the inhibition
of InsP$_3$R-type CRCs, identified in *Paramecium* by Ladenburger et al. (2006). These parasites encode PLC, and they react to exogenous InsP$_3$, whereas InsP$_3$R-type CRCs could not be identified as yet (Docampo et al., 2014; Garcia et al., 2017).

**FLASHBACK: MYTHS AND FAIRYTALES ABOUT CILIATES**

To give examples of unjustified hypotheses derived from misunderstood ciliate biology, let us begin with incredible stories, which one now may perceive as strange fairytales. This early view may have been blurred by the emotional attention early researcher have paid to readily available, beautiful, and agile organisms such as ciliates. One fama is the stubborn claim in the trivial literature, maintained by some people up to the 20th century, that there would take place a spontaneous generation of ciliates and some other “lower organisms” in hay infusions (Figure 1). Actually, the “generatio spontanea” of organisms had been defeated experimentally already in 1668 by the Italian Francesco Redi and, and with some delay, by the French writer and philosopher Voltaire (1751). Both had rejected the generatio spontanea hypothesis, before the German pathologist Rudolf Virchow in 1855 formulated the dictum “omnis cellula e cellula” (Each cell comes from another cell). The hay infusion myth was so stubbornly maintained that Klaus Hausmann and Willi Foissner († 2020) still in 1986 (!) felt like eradicating this fairytale once for ever in an essay (Hausmann & Foissner, 1986). For *Paramecium*, this was particularly strange since these cells do not produce cysts.

Yet, there was still another story pursued up to the early in the 1960s. The fairytale, as told in lectures by a zoology professor up to the 1960s, goes as follows: Once upon a time, there was a powerful protozoan—a ciliate. Ciliates were considered as progenitors of *Turbellaria acoela* and, and from thereon, of all metazoans. Both ciliates and these lowest level worms (Platyhelminthes) have a ciliated surface. Before the availability of the electron microscope, it appeared to some people that *Turbellaria acoela* would not be composed of cells, but rather represent a plasmodium, at least over large parts of the body. Moreover, upon staining with silver salts, introduced in the 1920s and 1930s as a diagnostic tool, ciliates display a “silver line system”; at that time, silver salts were known to stain neurofilaments, as summarized by Gray and Guillery (1961). Figure 3A shows an example of such preparations, prepared ~50 years ago by Willi Foissner when he found, by parallel electron microscopy, that the stained material is rather heterogenous, mostly located at the outlines of alveolar sacs; this includes reaction product in linear arrangement along ciliary basal bodies. Figure 3B was provided by Sabine Agatha demonstrating the current use of silver stain for systematic (and consequently also for ecological) purposes. From such early observations, the keen hypothesis had been launched that unicellular ciliates would contain precursors of neuronal structures, particularly since the silverline system...
connects cilia and thus was considered as a regulator of metachronous ciliary beat.

All this was condensed to the intriguing hypothesis saying that, during evolution, a “cellularization” process (subdivision into cells) would have transformed a ciliated protozoan into a predominantly plasmodial worm and further on into multicellular organisms. This means that the cytoplasm around each of the nuclei would have been delineated to form individual cells. In the 1920s and 1930s, this far-fetched hypothesis was supported by many researchers, though it was rejected by some zoologists already early on. Up to 1957, there was a heavy dispute between the exponents about pro and contra this hypothesis. In 1961, the opponents tried to find a final solution in an article designated as a “final discussion” (entitled “Schlusswort zur Diskussion Remane-Steinböck”). The “cellularization” fairytale was vigorously rejected by zoologists led by Adolf Remane and Peter Ax (Ax, 1961). Nevertheless, it was supported by some up to the early 1960s, as documented in an article by Steinböck (1963).

In his book on “Animal Evolution,” Nielsen (2012) summarizes: “…..The…”cellularization” theories, which derive a turbellariform-metazoan ancestor through metachronous ciliary beat. Thus, it was established that infraciliary lattice fibers are not involved in ciliary beat regulation, but it was all based on ion currents. All this helped to overcome old myths, while it heralded a new era of ciliates as model systems. The contribution by M. Valentine and J. Van Houten in this JEM issue summarizes current knowledge.

FROM SCIENTIA AMABILIS TO LABORATORY WORK

After its first description by Christian Huygens, 1678 (Dobell, 1932), Paramecium was illustrated by Louis Joblot in 1718, as reproduced in Figure 2. Due to its form, it was baptized “chausson” (slipper, Pantoffel; Joblot, 1718). Since then, it advanced to a favorite subject of light microscopists.

Ciliates move vividly, and all the sudden, they change swimming direction. During observation in the microscope, a ciliate, though unicellular, is always instinctively addressed as an animal, in German “Tiere” (e.g., Pantoffeltiere, and beasts by English- and French-speaking colleagues, respectively. Although this tendency to scientia amabilis was not always appreciated by every researcher, there were lots of cell biological phenomena to be demonstrated in college classes: extremely rapid secretion of secretory organelles (ejectosomes, trichocysts), galvanotaxis, ciliary beat and its acceleration or reversal, sexual activity, reproduction without sex, and allegedly eternal life by ongoing division. More detailed analyses have revealed that cells from a clone cannot survive infinitely. Here, Nobuyuchi Haga outlines insights from his painstaking microinjection studies including restricted life span of cloned paramecia. Formation of two types of nuclei, generative and vegetative, was considered as anticipating germine and soma. (Consequently, Hausmann & Bradbury, 1996 chose the title “Ciliates—Cells as Organisms” for a book.) But over decades, it remained enigmatic how these phenomena would be regulated, as discussed below.

Deciliation also allowed electrophysiologists to localize voltage-dependent Ca²⁺ channels, for a Ca²⁺-carried action potential, in cilia (Ogura & Machemer, 1980). Isolated ciliary membranes enabled the characterization of subunits of the voltage-dependent Ca²⁺ channels (Lodh et al., 2016). (Remarkably, these channels are no more bound to cilia in metazoans, as summarized previously [Plattner, 2017]). J. Van Houten and her associates (M. Valentine and J. Yano) present details concerning ciliary beat regulation; some of the mutations cause severe developmental defects in mammals.

Natural and induced mutations in ciliates have paved the way for the analysis of different cell functions, from exocytosis to ciliary beat. In many other cases, the method of complementation cloning by microinjection has proved important for identifying aberrant proteins in ciliates. Such complementation experiments had also been initiated by Haga et al. (1983), as well as by Keller and Cohen (2000). This has been

A way out from myths

Advanced research counteracted and compromised the “cellularization” chapter already during my first years of biology studies. First, EM analysis revealed that Turbellaria acoela are largely organized in cells, rather than as a plasmodium (Klima, 1967). Second, the unforgettable Willi Foissner († 2020), before raising to a form, it was baptized “chausson” (slipper, Pantoffel; Joblot, 1718). Since then, it advanced to a favorite subject of light microscopists.

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complemented by homology-dependent genetic inactivation (post-transcriptional gene silencing), by Meyer and Cohen (1999) and others, on the basis of a newly developed indexed genomic library for \textit{Paramecium tetraurelia}. New methods had to be developed, and new basic aspects could be learned, which we could never dream of at the time our work was started. Examples are presented in Figures 4 and 5. In part, it was facilitated by the establishment of sterile (axenic) cultures. Sterile mass cultures were more readily achieved, though, with \textit{Tetrahymena}. Here, stages of endo-/phago-lysosome organelles could be isolated by the uptake of ironedex-tan for magnetic separation of phagosomes (Vosskühler & Tiedtke, 1993). Isolated phagosome fractions from \textit{Tetrahymena} allowed for proteomic analysis of their membrane (Jacobs et al., 2006). For a long time, with \textit{Paramecium}, original recipes for axenic culture media (i.e. without food bacteria) were very difficult to set up and to handle. A breakthrough was achieved by Edna Kaneshiro (Kaneshiro et al., 1979). This provided clean samples, for example, for biochemistry work. Cilia and trichocyst contents were the first structures to be isolated, successively followed, by trichocysts with their membrane envelope and the trichocyst membranes came next, followed by pure fractions of other components. Cell surface complexes became available in great quantity (Lumpert et al., 1990; Vilmart-Seuwen et al., 1986). Surface complexes called “cortices” contained parts of the cell membrane with alveolar sacs and trichocysts still attached. Such fractions served as model systems for analyzing exocytosis in vitro, the kinetics of Ca\(^{2+}\) uptake and release (Länge et al., 1995; Mohamed et al., 2003), as well as for testing second messenger and specific toxin effects. Thus, analyses in vivo and in vitro could supplement each other. It took some time until macro- and micronuclei (MACs, MICs) could be differentially isolated by cytometry sorting, thus allowing for characterization of germline and macronuclear DNA (Guérin et al., 2017).

\textit{Tetrahymena} was more easy to maintain in sterile cultures and—as found later on—it has a restricted number of paralogs (Eisen et al., 2006) when compared to \textit{Paramecium (tetra)urelia}. Here, a great number of paralogs were found, called ohnologs to indicate that they were formed in the course of severalfold whole-genome duplications (Aury et al., 2006). This disadvantage also entails some advantages, however, since \textit{Paramecium} allows for the study of differential retention of ohnologs (McGrath et al., 2014) and their eventual neofunctionalization, for example, of tubulins.

Painstaking microinjection and microsurgery experiments were important for gene complementation studies (Haga et al., 1983; Keller & Cohen, 2000). In his article, Nobuyuki Haga makes us familiar with the capabilities of microinjection, including molecular complementation studies, showing details of MIC and MAC maturation and of clonal aging. Finally, constructs containing sequences interfering with endogenous mRNA were introduced by feeding, not only for genetic complementation but also for post-transcriptional gene silencing by interference (Galvani & Sperling, 2002; Ruiz et al., 1998). A combination of all these widely different methods, together with improved immuno- and affinity labeling and genetic labeling with green fluorescent protein (designated GFP, below), became a powerful tool in correlated structure–function analysis, also in ciliates.

In fact, mere electron microscopic analysis of ultrastructure in the second half of the past century had only been the prelude to more profound functional and localization studies envisaging dynamics, for example, of membrane trafficking. Analysis of trafficking of specific endo-/phago-/lysosomal organelles was enabled by a selection of monoclonal antibodies (Allen & Fok, 2000). From then on, with the intensification of molecular biology studies, improved light and electron imaging methods have been developed; these methods greatly helped to localize specific translation products and to establish their dynamics, not only in \textit{Tetrahymena} but also in \textit{Paramecium} (Guerrier et al., 2017; Plattner, 2017).
In ciliates, sexual activity by conjugation was described by O. F. Müller (1786) as follows: “Lusum praeterea duorum individuum, adultis cohabitantibus duplo breviorum, paucis minutis contemplatus sum” (roughly: … two individuals came together, as I observed over a few minutes…). This was followed up only a century later in two more detailed descriptions, both in the same year (Hertwig, 1889; Maupas, 1889). Conjugation includes division of the micronucleus and exchange between cells, followed by the formation of a new macronucleus. The view of these two authors was commented by Gruber (1889) in the sense that the designation of micronucleus and macronucleus means a generative and a metabolic nucleus (“Geschlechts- und Stoffwechselkern”), respectively, thus suggesting a development comparable to the
generative and somatic cells in metazoans. (This may have stimulated later on the “cellularization” hypothesis mentioned above). At that time, it may have been clear that the MIC is diploid, but the several hundredfold poly-ploidy of the MAC, for example, in Paramecium tetraurelia, had not yet been established. Early on, chromosomes and the mitotic spindle were described in Stylonychia by Bütschli (1876). As outlined below, it took almost half a century until it was disclosed by molecular biology that formation of a new micronucleus depends on information from the old macronucleus (which obliterates). In this issue, F. Drews, J. Boenigk, and M. Simon explain, in a well-illustrated survey, the epigenetic basis behind these processes. We also learn about current views of nuclear architecture and chromosomal fragmentation with its unsurpassed variability in ciliates, as reported here by R. Ahsan, W. Blanche, and L. Katz.

Before genomic analyses provided a new starting point for the ciliate community, several colleagues already achieved important results—just to mention the work by Glenn Herrick and Lawrence Klobutcher about genome modification during the micronucleus formation of a new micronucleus depends on information from the old macronucleus (which obliterates). In this issue, F. Drews, J. Boenigk, and M. Simon explain, in a well-illustrated survey, the epigenetic basis behind these processes. We also learn about current views of nuclear architecture and chromosomal fragmentation with its unsurpassed variability in ciliates, as reported here by R. Ahsan, W. Blanche, and L. Katz.

A BIG LEAP: FROM A SOLID BASIS TO CILIATE MOLECULAR BIOLOGY

From the year 2000 on, a turning point was reached with the international genome projects for Paramecium tetraurelia (Aury et al., 2006; Dessen et al., 2001) and Tetrahymena thermophila (Eisen et al., 2006). Together with other colleagues, Linda Sperling and Ed Orias were leading initiators for the Paramecium and Tetrahymena genome project, respectively. Indexed genomic libraries were set up for T. thermophila (Stover et al., 2006) and Paramecium t. (Arnaiz et al., 2007, 2012; Keller & Cohen, 2000), found at www.ciliate.org and https://paramecium.12bc.paris-saclay.fr, respectively. Together with expanding expression libraries for both species, they became an important source for molecular and cell biology studies. This enabled identification of key molecules for signal transduction, vesicle trafficking, biogenesis of organelles, etc. A variety of phenomena could, thus, be addressed, as summarized throughout this JEM review collection. It includes chapters on membrane dynamics and Ca++ signaling in Paramecium by this author. As mentioned, A. Soh and C. Pearson specifically address the epigenetically controlled optimization of the arrangement of basal bodies and cilia. Particularly, intriguing aspects concern molecular details of pattern formation specifically along the anterior/posterior axis during cell division, as scrupulously discussed in Tetrahymena by Eric Cole and Jacek Gaertig in a long article dedicated to Joseph Frankel, a pioneer of such challenging work. Many aspects still await dissection by molecular and genetic methods.

During evolution, like other organisms, ciliates have been invaded by foreign genetic material, for example, from bacteria, resulting in occasional integration of foreign DNA sequences in the nuclear genome. A mechanism to get rid of such noncoding sequences during the life of ciliate cells, they have developed a defense mechanism. This was achieved by excision of the foreign sequences during macronucleus formation (Klobutcher and Herrick, 1995); therefore, they were called “internal eliminated sequences” (IES). The process requires a scanning RNA (scnRNA), which compares sequences from the MIC and the old MAC to eliminate IES during the formation of a new MAC (Swart et al., 2014). In their contribution, M Simon and his colleagues illustrate mechanisms of crosstalk between MIC and MAC. The mechanism is different from the excision of introns in ciliates and also from the CRISPR/Cas mechanism to eliminate foreign DNA sequences detected in bacteria.

WHAT DRIVES EVOLUTION OF CILIATES?

Based on 16S/18S rRNA, Woese et al. (1990) aligned evolutionary pathways in bacteria, metazoans, and plants. Henceforth, bacteria were subdivided into eubacteria and into archaebacteria (Archaea). Later on, some evolutionists divided protozoa in monokonts and bikonts (Cavalier-Smith, 2003) depending on the number of basal bodies and cilia/flagella occurring either individually or locally in pairs. It was proposed that monokonts, from choanoflagellates on (like Monosiga brevicollis), developed to metazoans (King et al., 2008), whereas bikonts (from a level of the complex limnic Characaea on) were situated at the base of higher plants. The evolution of higher Monokonta can be traced much more convincingly than that of Bikonta, where ciliates, together with other phyla, form a more diffuse group of further evolution (Katz, 2012). At the molecular level both mono- and bikonts share many similarities (Plattner, 2015a). Therefore, whereas choanoflagellates are unique in important aspects, such as possession of tyrosine kinase activity beyond nuclear processes just as in metazoans (King et al., 2008), the situation is less clear for bikonts.

Intrinsic variation together with external ecologic forces can result in genetic drift and species formation (Lynch et al., 2011). A survey of large-scale evolution-based systematics of ciliates was presented a decade ago by Adl et al. (2012 and up to recent). As mentioned, Cavalier-Smith (2003) has argued in terms of mono- and bikont evolutionary lines. It is important to compare such views with results from phylogeny-based genomic databases or with analysis of small ribosomal rRNA sequences in different clades of ciliates (Boscaro...
increasingly feasible to compare \( \text{Ca}^{2+} \) properties including an action potential (though carried by Verkhratsky, 2013, 2018). Already in the 1970s, it became summarized in recent reviews (Plattner, 2017; Plattner & Verkhratsky, 2013, 2018). Already in the 1970s, it became increasingly evident that many molecules are maintained from ciliates up to mammals, including our neuronal and immunological system, as summarized in recent reviews (Plattner, 2017; Plattner & Verkhratsky, 2013, 2018). Already in the 1970s, it became increasingly feasible to compare Paramecium cells with neurons, not only because of electrophysiological properties including an action potential (though carried by \( \text{Ca}^{2+} \)) but also for similarities at a molecular and a functional level. This includes \( \text{Ca}^{2+} \) influx via voltage-gated \( \text{Ca}^{2+} \) channels, a range of other influx channels and intracellular \( \text{Ca}^{2+} \) release channels, plasmalemmal and intracellular \( \text{Ca}^{2+} \) pumps, \( \text{Ca}^{2+} \)-binding proteins such as calmodulin, SNAP proteins of the membrane fusion machinery, Rab-type GTPases and H\(^+-\)ATPase/pump for acidification of vesicle lumen, Ser/Thr-protein phosphatases such as PP2B (calcineurin, CaN), and second messenger-activated kinases (for summary, see Plattner and Verkhratsky, 2018). This comparison was most recently taken up by neurobiologist R. Brette (2021) in an article entitled “…Paramecium, a “swimming neuron”. Interestingly, some proteins are translocated within the cell; for example, \( \Delta V \)-sensitive \( \text{Ca}^{2+} \) channels are found only in cilia of ciliates and transferred to the cell membrane of neurons (Plattner & Verkhratsky, 2018). Altogether, some molecules may occur in bikonts and also in the evolutionary line from monokonts up to the highest level of animals. A further example is the maintenance of a dimeric protein phosphatase 2B from ciliates (Fraga et al., 2010) up to mammalian immune cells where it is responsible for rejection of organ transplants. An alternative is the selective maintenance of the modulatory subunit of PP2B in plants as “CaN-B-like protein” (Edel & Kudla, 2015). So, evolutionary cell biology shows some degree of preservation and of modification in the two evolutionary lines.

These examples illustrate that the mono-/bikont boundary is not strict or not well established. While many proteins are maintained in all systems, from protozoa to mammals and higher plants, respectively, this is not strictly the case with many proteins. Sources of diversification can originate in the production of alternatively mRNA forms, but alternative splicing is rare in Paramecium. Splicing of germline DNA in Paramecium has been analyzed, for example, by Catania et al. (2013), providing “insights into the role of DNA splicing in creating potentially functional genetic innovations”. Furthermore, new genes may be acquired by horizontal and vertical gene transfer, allowing for major steps in evolution.

**Integration of foreign DNA**

How are the chances of integrating foreign DNA as a driver of evolution? Since some time already, some genes are well established viral derivatives. A most impressive example is the effect of the transfer of retroviral genes encoding membrane fusion proteins (syncytin) to mammals about 130 to 145 million years ago (Puttick et al., 2016). This mediated the fusion of the outer layer of placenta cells to a syncytiotrophoblast (Mi et al., 2000) as a placenta barrier, which shields the embryo against attack by immune cells. Considering the age of ciliates estimated as between 0.8 and one billion years (Dorrell et al., 2013; Parfrey et al., 2011) or older, about 1980 to 2200 million years according to small subunit rRNA analysis (Wright & Lynn, 1997), this would be roughly 10 to 20 times more time than available for virus-based evolution of a placenta barrier. Thus, there would have been ample time for viruses to interfere with evolution of ciliates, perhaps with reproduction pathways. This view is supported by the similarity of fusogenic proteins of the superfamiliy of Fusexins, such as HAP2, from some viruses (Rubella, Dengue virus), ciliates (conjugation of ciliates; Cole et al., 2014) and other protists, up to gamete fusion in mammals (Brukman et al., 2022).

Evidently, similar interferences have occurred with bacterial genomes. Ciliates have taught us about defense mechanisms. This takes place by controlled deletions of internal eliminated sequences, IES, during formation of a new MAC, as detected in Paramecium (Klobutcher & Herrick, 1995) and also found in Tetrahymena (Fass et al., 2011), as discussed below. IES represent transposons, which, in the words of Chalker and Yao (2011), entail “domestication and genome surveillance”. It is interesting to see, from ciliates to mammals, what kind of compromises cells had to make for achieving increasing functional and structural complexity. In this JEM issue, the team C. Timmons, S. Shazib, and L. Katz deals with the transfer of viral and bacterial gene derivatives in ciliates. These authors also argue that nuclear dimorphism in ciliates may originate from bacterial transposons, which were co-opted in the genome for programmed deletion of DNA (introns). Bacterial endosymbionts may have adapted the genome of ciliate in ruminating animals, enabling them to develop new metabolic pathways. Johana Rotterová and associates (Virgina Edgecomb, Ivan Čepička, and Roxanne Beinart) present anaerobic ciliates as models for the evolution of life in anoxia. They also address the formation of methane by ciliate symbionts—an aspect of paramount importance in current discussions about global warming.
In summary, during evolution, we see old components and mechanisms, fully maintained or varied, together with innovations. This also applies to ciliates, as documented for several aspects in this JEM special issue. Therefore, the existence of nuclear dimorphism was a challenge and an opportunity at the same time. In the 2010 years, it was recognized that the elimination of IES is not always absolutely precise; it is both precise and imprecise in *Paramecium* and more precise in *Tetrahymena* (Allen & Nowacki, 2017). More scrutinized analyses revealed that some imprecision in IES handling, particularly with nucleotide shifts, may yield a playing mass for evolving new genes (Vitali et al., 2018). There are also some differences in the programmed genome rearrangement in different ciliates (Rzeszutek et al., 2020) some of which wait for more profound analysis.

**CILIATES—A PRECIOUS MODEL FOR EPIGENETICS**

In a classical view, several main mechanisms are (i) structural transformation of a protein's secondary structure (Prion proteins, PrP), (ii) covalent modification of DNA and of histones, and (iii) the interference of non-protein coding RNAs. (iv) Integration of viral or bacterial DNA and of histones, and (iii) the interference of non-protein (Prion proteins, PrP), (ii) covalent modification of DNA and acetylation and DNA methylation. In addition, a plethora of noncoding RNA species of widely different types has subsequently been detected. Altogether, DNA and histone modifications and noncoding DNA can regulate gene transcription in the positive or negative sense, as well as selective formation of splice variants by long noncoding RNA. This is the view achieved on the background information from higher eukaryotes, as outlined by Allis and Jenuwein (2016) and Harvey et al. (2018); they provide a survey of general aspects in animal cells, while Gozmanova et al. (2017) describe this for plant cells.

Early on, histone acetylation has been reported from *Stylonychia* (Lipps, 1975). Brownell et al. (1996) described the RNAi-dependent effect of histone acetylation, and Liu et al. (2007), a similar effect of histone methylation. Most recently, several groups have summarized the effects of such histone modifications and of noncoding RNA species on DNA rearrangement. In the present JEM issue, M. Simon, F. Drews, and J. Boenigk provide impressive illustrations of various kinds of epigenetic effects in ciliates, based on experience with *Paramecium*.

Various aspects of epigenetics in *Paramecium* and *Tetrahymena* have been followed up in work by Eric Meyer, Mariusz Nowacki, Eduardo Orias, and others, as summarized by Orias et al. (2017). This involves a variety of phenomena studied in laboratories dedicated to ciliate epigenetics. MIC → MAC transformation in the course of sexual activity (conjugation or autogamy) is epigenetically regulated; this transformation takes place on the basis of information from the MIC, via the old MAC, involving release and trimming of scanning RNAs, which thus determines the exclusion of IES in the MIC destined to become the new MAC (Meyer et al., 1997). However, long before genomic research in ciliates had achieved an adequate level, non-Mendelian expression of surface antigens had been described in *Paramecium* (Sonneborn, 1949). Later on, variant surface antigen expression became seminal for the understanding of host cell invasion by Apicomplexan parasites, which change their surface proteins too fast to allow for any immunological reaction by the host. What is the importance of work with ciliates?

Any input of epigenetic phenomena to evolution, in the extreme sense of a Lamarckian hypothesis, that is, inheritance of acquired characteristics, is almost unequivocally denied in the literature. However, does the
integration of foreign gene sequences during evolution encompass epigenetic processes? What drives evolution in ciliates altogether? The aforementioned invasion by viral and bacterial gene sequences into genomes, coupled with effects on ciliate genome architecture and expression, is another epigenetic pathway (C. Timmons et al., this issue). Variation in IES elimination can be one of the driving forces. The latter mechanism is much less precise in *Paramecium* than in *Tetrahymena* (Allen & Nowacki, 2017) and, thus, may potentially drive evolutionary changes (Arnaiz et al., 2012). One single event of imprecise IES excision may be passed to progeny (transgenerational epigenetics) where it could be of potential advantage for the cell.

Whereas the elucidation of mechanisms of transgenerational epigenetics in ciliates progressed (Chalker et al., 2013; Meyer & Duharcourt, 1996), the occurrence of this phenomenon is still intensely debated in metazoans (beyond nematodes) up to humans. Generally, sociologists support its occurrence—opposite to most mammalian biologists who emphasize the imponderabilities of intrauterine influence during pregnancy (Hagshemke, 2018; Van Otterdijk & Michels, 2016). What may make the difference and which is the situation in ciliates?

One of the main differences between ciliates and metazoans may be the persistence of histones during the MIC → MAC cycle, with various modifications in both nuclei. Acetylation of micronuclear histones during conjugation has been detected in *Tetrahymena* already in 1985 (Allis et al., 1985). In contrast, mammalian spermatooza dismantle chromatin by exchanging histones for protamines. Therefore, ciliates have advanced to precious models for transgenerational epigenetics (Chalker et al., 2013; Duharcourt et al., 1995; Meyer et al., 1997; Meyer & Duharcourt, 1996)—one of the most fascinating phenomena of current research in molecular biology. One question currently debated is whether in ciliates newly acquired responses to ambient parameters may be transmitted epigenetically from generation to generation in ciliates. F. Catania’s group has induced phenotypic changes by exposing *Paramecium* to unusual culture temperatures (Hagen et al., 2020). In cautious wording, the authors suggest the possibility of transgenerational genetic fixation of induced changes.

Both metazoan germline cells and ciliates possess Piwi-(P-element induced wimpy testis) proteins for gene regulation (Fang et al., 2012; Mochizuki et al., 2002). Piwi proteins interact with noncoding RNAs (Ross et al., 2014). Recently, research with mammalian cells has detected the transfer of piRNA (piwi-interacting RNA) by small vesicles from cells of the epididymis to maturing spermatozoa (Machtinger et al., 2016; Sharma et al., 2018). May this open up new aspects of transgenerational epigenetics up to mammals? Furthermore, which mechanisms found in ciliates may further be detected in mammals and vice versa?

In this context, let us look back onto the first type of epigenetic mechanisms, that is, aberrant configurational changes of proteins, mentioned at the beginning:Remarkably infection with PrPSc is also assumed to take place via microvesicles released by exocytosis from multivesicular bodies (Aguzzi & Rajendran, 2009). These organelles have recently been praised as rising stars of cell biology (Pluchino & Smith, 2019), yet we have only scant knowledge about the role of membrane bounded microvesicles in ciliates during mating (Cole et al., 2015). Altogether, there is considerable movement in this novel branch of research, with ciliates acting as key players and confrontation with open questions up to humans.

**SYMBIOSIS, EXTRA- AND INTRACELLULAR SIGNALING**

**Ciliates as models for symbiotic integration of foreign microorganisms**

Since a long time, the endosymbiotic origin of mitochondria and chloroplasts is well established. However, recently all the sudden an unexpected aspect arose from in vitro fertilization in a family with a mitochondriopathy. Normally, one would not give much attention to the exchange of mitochondria in different *Paramecium* strains. Transfer of mitochondria revealed restricted compatibility, as became manifest by degradation (Sainsard-Chanet & Cummings, 1988). This aspect should have received public attention 25 years later when Amato et al. (2014), in the course of in vitro fertilization, intended to override a defect in the respective mtDNA-encoded gene. Consequently, in the course of in vitro fertilization, mitochondria (strictly maternally transmitted in mammals) were replaced by mitochondria from a healthy donor. This type of gene replacement therapy results in babies with three parents. Any incompatibility problems were not mentioned and neither were results with *Paramecium* cells. At this time, mitochondrial transplantation is very much under debate as a possible therapeutic (Lightowlers et al., 2020).

Here, Sergei Fokin and Valentina Serra present a survey of widely different bacterial symbioses in ciliates. Also, in this volume M. Fujishima and Y. Kodama delve into mechanisms of how such secondary endosymbioses are established in ciliates; therefore, they expect to recapitulate basic aspects of the evolution of endosymbiotic organelles by primary endosymbiosis.

**Social life of ciliates**

Ciliates have served as models for sensory transduction of electrical, chemical, and mechanical stimuli, including graviperception and activity of gamones and kairomones. Already more than two centuries ago,
Müller (1786) noted that *Paramecium* reacts to electrical and magnetic stimuli (“attractioni electriceae & repulsioni magneticae”). Jennings (1906) published a book, still worth reading, on the “behavior of the lower organisms,” containing careful observations with *Paramecium*. Response to an electric field allows one to command *Paramecium* cells to swim in one direction, which changes when the polarity of the field is reversed. Once electrophysiology became established, behavior could be correlated with different ion currents, based on manifold cation channels in the cell membrane, including some reacting to hyper- or depolarization, some mechanosensitive (tension), and still other channels. Based on electrophysiology, ciliary beat regulation could be elucidated (Eckert & Brehm, 1979; Eckert & Naitoh, 1972; Machemer, 1988). Further on, the effects of chemical attractors and repellents have been analyzed (Van Houten et al., 2000). On this basis, new insights into mechanisms of graviperception have been obtained (Hemmersbach et al., 2005; Krause et al., 2010; Machemer, 2014). Here, M. Valentine and J. Van Houten outline the sequential activity of somatic and ciliary channels, involving the cooperation of receptor potential (generated over the somatic membrane) and a Ca$^{2+}$-based action potential over the ciliary membrane. Kairomones for extracellular communication have been detected in a number of ciliates since the work by Kusch and Heckmann (1992). Kairomone effects have been shown to be involved in producing micro-/macrostome forms in *Tetrahymena vorax* (GrØnlien et al., 2011)—clearly an epigenetic effect requiring further analysis. Research on gamones for sexual signaling and attraction, resulting in interaction with their respective receptors, has been combined with molecular structure analysis (Luporini et al., 2014).

During evolution, members of some ciliate species learned to communicate with each other in different ways of an intraspecific cross-talk. It was in July 1981 in Warsaw that I heard for the first time Piero Luporini talk about mating in *Euplotes*—a line he consistently pursued since then. (The meeting was overshadowed by the impending invasion of Soviet troops, which fortunately did not take place.) In addition, together with their associates (C. Alimenti, F. Buonanno, G. Di Giuseppe, G. Guella, C. Ortenzi), Piero Luporini and Adriana Vallesi include the release of bioactive molecules in this volume. They address protein pheromones/gamones, terpenoids, such as “euplotins” suggested to serve for interspecies communication and also other compounds that are stored as secondary metabolites in extrusomes of some species. For part of the work, Piero Luporini was able to win Kurt Wüthrich (Zurich, Switzerland, Nobel Prize in Chemistry 2002) for analyzing receptor–ligand interaction by nuclear magnetic resonance spectroscopy. The result was a molecular fit of pheromones and their receptors in a key-lock manner. Altogether, it was thus demonstrated that ciliates under scrutiny already possess a defined spectrum of sensory perception, which, in principle, reaches up to mammals. In this context, as stated already, Piero Luporini advanced to a reporter of affaires amoureuses of ciliates.

Ciliates as models in classical and molecular cell biology of intracellular dynamics

Prerequisite for deciphering signals and pathways of vesicle trafficking in molecular details was the basic work by Allen and Fok (2000). They have scrutinized various branches of endocytosis and phagocytosis by immunostaining with monoclonal antibodies and additionally by applying fluid markers at the electron microscope level. Once molecular biology was established with ciliates, this was the starting point for the identification and localization of specific proteins from about the turn of the century on. Examples are presented in Figures 4 and 5, including immuno-light and electron microscopic labeling using antibodies obtained after prognosticating immunogenic epitopes, including specific antigen subtype labeling, that is, of ohnologs. Affinity labeling can also sometimes be appropriate to complement antibody labeling. For details, see legends to Figures 4 and 5. Such work includes localization of different Ca$^{2+}$ channels (Figure 5) and Ca$^{2+}$-ATPases/pumps (Figure 5A,B) starting from gene structures. Until then, with ciliates, such work had been a struggle, often frustrating and unreliable, when commercially available mammalian-based antibodies were applied.

One reason may be that ciliates, at an evolutionary level, are simply too different from higher eukaryotes. Therefore, the production of antibodies against prognosticated antigenic sequences became a convenient alternative to monoclonal antibodies. In addition, genetic labeling by tagging proteins with GFP was established (Hauser et al., 2000a; Hauser et al., 2000b). This has eventually been combined with anti-GFP antibodies at the light or electron microscope level. Figure 5C shows that cryosections, though methodically rather demanding, can localize proteins with highest sensitivity. Labeling is relatively easy to achieve with cell surface structures in vivo (pre-embedding labeling), but also, labeling of ultrathin sections can be rather sensitive (post-embedding labeling) when applied to materials embedded in appropriate media (Figure 5D). Another aspect is the combination of immuno- and affinity staining, respectively, with different color for simultaneous visualization of different cell components (Figure 5E). Moreover, in the cell shown, samples had been depleted of PrAct4 by post-transcriptional silencing; since this actin isoform normally forms the cleavage furrow (Figure 5G), the silenced cell in Figure 4E does not divide; rather, it grew thick and accumulates several contractile vacuole complexes as it would before cell division. Moreover, Figure 5F documents the inability of fluorescent phalloidin, the classical stain for F-actin, to stain the cleavage furrow in a...
Paramecium cell—a problem one experiences with many toxins. In summary, Figure 5E–G document the possibility to combine widely different methods, from genetic manipulation to widely different staining methods; they also warn from overestimating the reliability of localization studies in ciliates by affinity staining, as this depends on the knowledge of the ciliate-specific molecular and pharmacological background (Plattner et al., 2009).

It was a long way from the daring postulate of protein-regulated membrane fusion, first based on work with mammalian cells (see Rothman, 2014) and then expanded to Paramecium cells (Plattner & Kissmehl, 2003). Vesicle trafficking in ciliates, like in mammalian cells, was found to depend on SNARE proteins (soluble NEM-sensitive attachment protein receptors) (Plattner, 2010); previously, their mere existence in ciliates and other protozoa has not been considered as granted in the literature. This was complemented by the SNARE-chaperone NSF (Kissmehl et al., 2002) and the polymeric H^+)-ATPase/pump (Wassmer et al., 2009) whose subunit connecting the head and basal piece is present in an incomparable large number of ohnologs. This may account for locally different functional requirements, that is, not only directly for acidification of vesicle lumen, but indirectly also as a sensor for binding GTPase modifiers. These mediate the attachment of GTPases and, thus, progress of vesicle docking and fusion—an insight achieved with mammalian cells by Hurtado-Lorenzo et al. (2006). This is complemented by a list of Rab-type GTPases, as analyzed in Tetrahymena thermophila, complemented by data from Paramecium tetraurelia (Bright et al., 2010). All these components (and many more) are relevant for vesicle interaction and ultimately for membrane fusion in ciliates.

In principle, the osmoregulatory “contractile vacuole complex” is a secretory system with isoforms of the molecules just mentioned (Plattner, 2015b) and with aquaporin for facilitating sequestration and expulsion of H\textsubscript{2}O, together with ions, in consequence of osmotic pressure (Ishida et al., 2021). Accordingly, it had to be expected that the system would also contain microdomain-forming proteins of the type Reggie/Flotillin, which are prerequisite for the positioning of pressure- (mechano-)sensitive channels (Reuter et al., 2013), just like in mammalian systems. This organelle also contains Ca\textsuperscript{2+}, PLC of the type releasing InsP\textsubscript{3} (Staudt et al., 2016) and InsP\textsubscript{4}-activated Ca\textsuperscript{2+}-release channels (Ladenburger et al., 2006). (The main function of the contractile vacuole complex, however, is to expel an excess of Ca\textsuperscript{2+} and water.) It may be rewarding to use the complex methodology applied in this work for the identification of InsP\textsubscript{3} receptors in Paramecium also in Apicomplexan parasites where their occurrence is still unsettled (Docampo et al., 2014; Garcia et al., 2017).

Intracellular Ca\textsuperscript{2+} signals mediate vesicle/membrane interactions. Ca\textsuperscript{2+}-release channels of different types were identified and analyzed in Paramecium (Ladenburger & Plattner, 2011). Clearly, Ca\textsuperscript{2+} is a main second messenger also in ciliates, complemented by cyclic nucleotides and Ca\textsuperscript{2+}-binding proteins of different types: high sensitivity/low capacity and low sensitivity/high-capacity types for signaling and binding of high intracellular concentrations, respectively. Not all of these channels are of an orthodox molecular structure (Plattner, 2017; Plattner & Verkhratsky, 2013). Most intriguing is the cooperation of Ca\textsuperscript{2+} release from alveolar sacs coupled to secondary influx (store-operated Ca\textsuperscript{2+}-influx; Hardt & Plattner, 2000). InsP\textsubscript{4}-CRC channels also drive ongoing membrane fusions within the contractile vacuole complex and fusion of the contractile vacuole with the cell membrane. In this JEM special issue, an article of this author is dedicated to vesicle trafficking in ciliates and Ca\textsuperscript{2+} signaling in manifold roles.

### SPECIFIC PROBLEMS IN WORK WITH CILIATES AND OPEN QUESTIONS

Some “nasty” sides of ciliates

Unexpectedly, most of the classical inhibitor drugs fail in Paramecium and probably also in other ciliates. Classical examples are drugs directed against cytoskeletal elements, such as microtubules (Pape et al., 1991) and actin filaments, as reviewed on a broader scale (Plattner et al., 2009). Another disadvantage is that patch-clamp electrophysiology is not applicable to ciliates because of the size of the cells, their rather rigid surface, and the many cilia. Alternatively, whole-cell patching was successfully used for registration of Ca\textsuperscript{2+}/calmodulin-activated cation channels (Erxleben & Plattner, 1994) contained in the cell membrane of Paramecium (Preston et al., 1991; Saimi & Kung, 2002). Concomitantly, these signals could be used to indirectly monitor free Ca\textsuperscript{2+}-concentrations adjacent to the cell membrane. Such signals could be inhibited by anti-calmodulin drugs, as had been recognized already in electrophysiological recordings by Roger Eckert from the 1960s on (Brehm & Eckert, 1978)—a concept extended in the sense that calmodulin, in Paramecium, orchestrates membrane responses to stimuli (Kung et al., 1992; Preston et al., 1991).

Particularly, cumbersome problems arose in early attempts to register intracellular, cytosolic Ca\textsuperscript{2+}-dynamics with fluorochromes. These were not taken up easily and if so, when applied over longer times or using membrane-permeable derivatives, fluorochromes were sequestered in acidic organelles; attempts toward reliable F/F\textsubscript{0} ratio imaging initially also failed because of the agility of these cells, until my doctoral student Norbert Klauke tackled this problem by squeezing cells gently into the meniscus of a culture droplet (Klauke & Plattner, 1997), followed by injection of fluorochromes under inverted microscope control. All these technical problems may be
the reason why hardly any competition did ever arise in
Ca2+ imaging with ciliates.

A selection of open questions

It was a saga that the late Willi Foissner had just to stick his hands into the soil somewhere to find a new ciliate species. Surely, in terrestrial, limnic and marine ecology further investigations will still find new important key players and indicator species. This makes hunting for new types of the “last biktons” a useful and honorable meter.

In ciliate research, several serious questions remain open, also concerning their cell biology. There is only marginal evidence of the occurrence of a Ca2+/polycation sensing receptor in the cell membrane, relevant for exocytosis stimulation by polycationic compounds (comparable to degranulation of mast cells by compound 48/80 in mammalian mast cells). The same is true of trimeric G proteins for signal transmission from the cell membrane on. Though a fusion mediating Ca2+ sensor of the type synaptotagmin does occur in the Paramecium database, it contains an unusual number of C2 domains (unpublished), as confirmed by Thomas Südhof via email. Such enhanced synaptotagmin forms (eTags) also occur in some mammalian cells including brain (Min et al., 2007), in addition to a large number of isoforms with the orthodox number of two C2 domains (Südhof, 2002). Also, rather unresolved is the role of the login-type of synaptobrevins in ciliates, which originally was reported from plants (Uemura et al., 2004).

The topics virus–ciliate and bacteria–ciliate interactions still appear far from completed. Using barcoding, endosymbiosis research may discover many more bacteria- and virus-derived genome sequences nesting in ciliates, with potential health hazard during further evolution. Also, some details still to be detected with ciliates may have some bearing on malaria and toxoplasmosis research (Sparvoli & Lebrun, 2021).

CONCLUSIONS

Ciliates were among the first cells described and routinely observed in the light microscope. Soon genetic aspects, including nuclear dimorphism, conjugation and autogamy came into the focus. The career of ciliates in electron microscopy started with an analysis of Paramecium multimicronucleatum by Sedar and Porter (1955), the senior author having been known for having an eye on beautiful objects. How intriguing some structural details were to interpret at that time becomes evident from the fact that the authors have confused the inner alveolar sacs membrane with the plasma membrane (cell membrane). A wealth of localization methods has been developed, in parallel to molecular genetic methods. Successively epigenetic effects have been recognized in ciliates where they still are in the focus of research. All this made ciliates, notably Tetrahymena and Paramecium, attractive model systems to which we owe important insights of general importance.

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