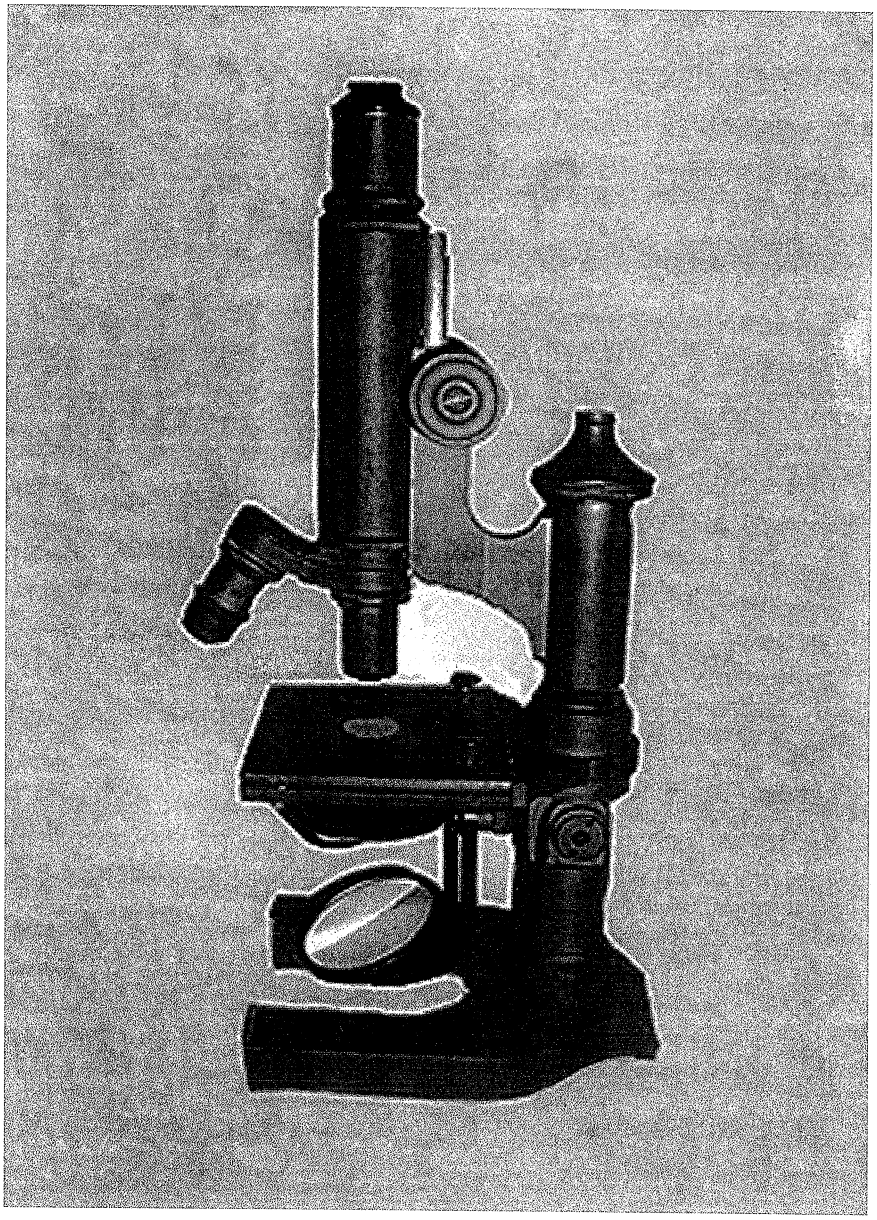
BEHIND THE DISCOVERY OF "NISSENBAUM'S FIXATIVE"

GERALD NISSENBAUM



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FROM THE EDITOR

There are some, like Arthur C. Clarke, who would argue that January 2001 is the beginning of the next Millennium. If you take this position, then the new Millennium for ***The Journal of Eukaryotic Microbiology*** is beginning with some significant firsts while the past Millennium is ending with some significant accomplishments.

You will notice that there has been a substantial turnover in the Editorial Board with the retirement of four Associate Editors: Kathy Karrer resigned in September while Roger Anderson, Don Lindmark, and Peter Satir retired as of December 2000. Together, these colleagues have served the Society of Protozoologists and ***The Journal of Eukaryotic Microbiology*** for a cumulative 40 years. On behalf of the Society and the Editors of the ***The Journal***, I gratefully acknowledge their dedication and interest. I am particularly thankful for the continuity that they provided during my first term as Editor-in-Chief. Please convey your personal thanks to them for a job well done! I am pleased that Jim Berger, Melanie Cushion, Larry Klobutcher, and Andrew Rogerson have agreed to replace them.

Last year, I commissioned the Institute for Scientific Information, Philadelphia, to create a database for articles published in ***The Journal*** between 1990–1999 so that I could investigate the influence that various types of articles were having in the field. I will be presenting a final report to the Executive Committee this year. Briefly, it is encouraging to see that Invited Reviews and Symposia average almost 30% more citations than Regular Articles. Thus, the Society's support of these publications is having a benefit. The three most-cited authors during this period were Ann Cali (Rutgers), Jan M. Orenstein (George Washington), and Govinda S. Visvesvara (CDC). Not unexpectedly, all three were among the authors of the top fifteen most cited articles (Table 1). You will note that nine of the articles are on the ''sporozoa'' sensu lato, five deal with phylogeny, two with infectious amoebae, and one derives from a 1991 ecology symposium. In 1991, articles from the International Workshops on Opportunistic Protozoa were comparable to Regular Articles in citations but by 1997 had dropped to below 50% the citation rate of Regular Articles. Overall, my conclusion is that the citation rate has remained stable over this period, confirming the strength of ***The Journal*** in the field.

Today, we are not only encouraged to use quantitative measures of the impact of our science, like citation rates, but we are also encouraged to explain the process and importance of science to the public. Science is often considered to involve a linear process of discovery, and certainly, there are many discoveries that lie at the end of a long road of perseverance. There are other, more apocryphal stories of serendipitous discoveries in the history of science: Kekulé's vision in the flames of his fireplace of a snake biting its tail and so inspiring the model for the structure of benzene, and Fleming's moldy plates providing the first clue to the existence of antibiotics. The recent example from the Nobel Prize for Chemistry 2000 provides yet another case: Hideki Shirakawa (Tsukuba) added one thousand-fold too much catalyst to his mixture and generated a beautiful thin film of the polymer *trans* polyacetylene! The lead article in this issue, and the beginning of a new series of articles entitled ''Behind the Discovery'' is the story of the development of Nissenbaum's Fixative. Some months ago the author's son contacted the Editorial Office to enquire about ***The Journal's*** interest in documenting his father's discovery in his student days. Gerald Nissenbaum agreed to tell his own story here. Dr. Nissenbaum's ''accidental spill'' led to a significant advance in fixation-staining techniques for protozoa. I believe these are important records to publish, not only to inform young scientists of the process of discovery in our own discipline, but also to provide to the general public a record of how science ''works''. I encourage you to provide me with names of more of these kinds of discoveries so that we may publish and record these personal accounts in our Journal pages.

Finally, you will have read in our recent Newsletter that the Executive Committee has agreed that the Society join the BioOne electronic publishing initiative, which is being technically supported by Allen Press. As you may know, institutional subscriptions for ***The Journal*** have been declining at a rate of about 5% per year for over five years. We all know that our libraries' budgets have been severely challenged during this period of time. It is hoped that ***The Journal*** will recapture some of these subscriptions by joining BioOne and being ''bundled'' with a large number of other significant journals. Our contract will be for two years at which time the Executive Committee will review the relationship and determine whether it is in our best interests to continue.

Although we may recapture institutional subscriptions through BioOne, it is important for each of us to consider reasons for maintaining our individual

Table 1. Top fifteen most cited papers in *The Journal of Eukaryotic Microbiology* between 1990–1999 inclusive. Data supplied by the Institute for Scientific Information, Philadelphia.

Cites	Authors	Title	Year	Vol.	Pages
169	A. Cali, D. P. Kotler, J. M. Orenstein	*Septata intestinalis* n. g., n. sp., an intestinal microsporidian associated with chronic diarrhea and dissemination in AIDS patients.	1993	40	101–112
107	A. Cali, R. L. Owen	Intracellular development of *Enterocytozoon,* a unique microsporidian found in the intestine of AIDS patients.	1990	37	145–155
101	L. S. Diamond, C. G. Clark	A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925.	1993	40	340–344
69	G. S. Visvesvara, G. J. Leitch, H.Moura, S. Wallace, R. Weber, R. T. Bryan	Culture, electron microscopy, and immunoblot studies on a microsporidian parasite isolated from the urine of a patient with AIDS.	1991	38S	105–111
64	D. J. Gifford	The protozoan-metazoan trophic link in pelagic ecosystems.	1991	38	81–86
64	C. R. Vossbrinck, M. D. Baker, E. S. Didier, B. A. Debrunner-Vossbrinck, J. A. Shadduck	Ribosomal DNA sequences of *Encephalitozoon hellem* and *Encephalitozoon cuniculi*: Species identification and phylogenetic reconstruction.	1993	40	354–362
58	G. S. Visvesvara, J. K. Stehr-Green	Epidemiology of free-living ameba infections.	1990	37S	25–33
53	J. R. Stringer, S. L. Stringer, J. Zhang, R. Baughman, A. G. Smulian, M. T. Cushion	Molecular genetic distinction of *Pneumocystis carinii* from rats and humans.	1993	40	733–741
50	M. P. Sherman, M. L. Loro, V. A. Wong, D. P. Tashkin	Cytokine- and *Pneumocystis carinii*-induced L-arginine oxidation by murine and human pulmonary alveolar macrophages.	1991	38S	234–236
44	M. D. Baker, C. R. Vossbrinck, E. S. Didier, J. V. Maddox, J. A. Shadduck	Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms.	1995	42	564–570
44	P. J. Didier, E. S. Didier, J. M. Orenstein, J. A. Shadduck	Fine structure of a new human microsporidian, *Encephalitozoon hellem,* in culture.	1991	38	502–507
43	S. J. Greenwood, M. Schlegel, M. L. Sogin, D. H. Lynn	Phylogenetic relationships of *Blepharisma americanum* and *Colpoda inflata* within the phylum Ciliophora inferred from complete small subunit rRNA gene sequences.	1991	38	1–6
43	J. Gunderson, G. Hinkle, D. Leipe, H. G. Morrison, S. K. Stickel, D. A. Odelson, J. A. Breznak, T. A. Nerad, M. Müller, M. L. Sogin	Phylogeny of trichomonads inferred from small-subunit rRNA sequences.	1995	42	411–415
42	A. Cali	General microsporidian features and recent findings on AIDS isolates.	1991	38	625–630
42	E. Viscogliosi, H. Philippe, A. Baroin, R. Perasso, G. Brugerolle	Phylogeny of trichomonads based on partial sequences of large subunit rRNA and on cladistic analysis of morphological data.	1993	40	411–421

member subscriptions. Electronic media are rapidly approaching printed media in regard to quality of reproduction, so that it is becoming much more difficult to rationalize continuing to receive a paper copy on those grounds. There will be a temptation to not renew individual member subscriptions if your library subscribes to BioOne as you will be able to read ***The Journal*** after several clicks of the mouse buttons. I am sure there are other reasons that one can find for not parting with those individual subscription dollars! However, I ask you to remember that the founders of our Society and ***The Journal*** believed that to advance protozoology these two "instruments" were necessary innovations. Our membership fees continue to perform a crucial function in supporting ***The Journal*** and maintaining the profile of our discipline in the broader community. I am encouraged that individual memberships appear to have stabilized in the last two years. Thank you for your continued support of the Society and ***The Journal*** in the coming years.

My best wishes for the New Millennium!

DENIS LYNN
Editor-in-Chief

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Behind the Discovery of "Nissenbaum's Fixative"

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ABSTRACT. The author describes the serendipitous discovery, conception, development, and history of Nissenbaum's Fixative while an undergraduate biology major in the early 1950s. The subsequent uses, applications, and modifications over the past forty-seven years are also described. Some of the modifications omitted from his short original paper are mentioned. Highlights of his subsequent career in the field of medicine are noted.

Key Words. Combined fixation and adhesion, history, inventions, serendipity.

WHILE attempting to recall remote events, the mind is like an ocean: murky, except for islands of remembrance that jut out and are visible. So it is in writing this paper about events leading to the development of a unique fixation method I conceived and developed almost fifty years ago.

As is often the case, I became interested in natural things as a young child, having had the opportunity to experience nature at summer camp in New Jersey. At the age of eleven, I started saving for a microscope. A year or so later, I purchased one, including accessories, for $12.00 from the A.C. Gilbert Company in New York City. I spent many hours going through the simple to complex exercises in its accompanying "*Exploring the World with a Microscope*" (Richards 1938), a manual that was quite extensive and detailed.

Upon entering high school, I joined the Biology Club and a whole new vista opened. Three or four of us would take an occasional morning off and rent a rowboat at Central Park Lake in New York City. We carried containers and sampled the water and sediment in various parts of the lake. These were brought back to the school and the organisms were more or less identified under the microscope, followed by attempts to culture the specimens (mostly unsuccessfully). I recall being especially fascinated by the movement and antics of rotifers.

By the time I was a high school junior, we had progressed to more advanced projects with protozoa, ranging from staining to in vitro trophic experiments. That year, as President of the Biology Club, I found a large, unused walk-in refrigerator for the Club to use. We installed shelving and a workbench, and borrowed a monocular microscope from the main high school lab. This satellite lab allowed us to come and go at all hours of the day and night. Our mentor was Mr. Edward Frankel, Chairman of the Biology Department, who encouraged us and supplied reagents and glassware. Our reference library consisted of second-hand books—*Biology of the Protozoa* (Calkins 1926); *Working with the Microscope* (Corrington 1941); and *Animal Micrology* (Guyer 1936).

The high school was loosely associated with Yeshiva College, all a part of the larger University, with its graduate schools. Thus, I met and became friendly with the Chairman of the Biology Department, Dr. Meyer Atlas. He had taught at the college since the 1930's and also conducted his own research. We discussed a project I had in mind to duplicate the results of Lund's (1933) work on the neuromotor system of *Paramecium caudatum.* He guided me in learning the silver stain of Klein, Klein's method with Hayes' modifications, and my own modification of Gelei and Horvath's method as described by Lund (1933).

Dr. Atlas gave me lab space in a corner of the college laboratory, consisting of a lab bench with multiple drawers and small built-in cubby for a microscope, adjacent to gas outlets and a sink. In return, I performed some lab chores, including keeping the amphibian tanks clean and preparing media for *Drosophila melanogaster* genetics. I had to coordinate my visits there with his schedule, since the microscopes were locked in a safe in his office. Thus, I worked between the walk-in refrigerator lab at the high school and the college lab. I preferred the latter since I could show Dr. Atlas my silver stains and had access to his library.

In 1949, while a high school junior, Mr. Frankel asked me to prepare an exhibit of the neuromotor system for presentation at the Federation of Science Teachers of New York City. The meeting was held at the Waldorf Astoria Hotel as part of the convention of the American Association for the Advancement of Science (AAAS). I demonstrated my best slides, using two microscopes, and distributed a paper. The first formal meeting of The American Society of Protozoologists was held at that AAAS meeting in 1949 (Corliss 1998).

Upon graduation from high school, I received the Bausch and Lomb Science Award and entered Yeshiva College, majoring in biology and chemistry. I continued to study the fibrillar system in my corner lab where I had access to the laboratory and my work space at any time. However, as previously noted, the microscopes were locked away in a safe and could be accessed only when Dr. Atlas was available. To solve the problem, I decided to look for a used second-hand microscope.

I visited August E. Waeldin Optical Company at 10 Maiden Lane in the Wall Street area. The salesman had a wide array of used microscopes and asked me in what price range I was interested. I told him $25.00. He looked at me for a few moments and said, "I think I have something in the back." He brought out a dusty, old Leitz monocular microscope with two objectives but missing the coarse adjustment, sub-stage condenser, and diaphragm. He offered it to me for $10.00, and I bought it.

During cleaning, I found that after I wedged a thin, wooden combustion stick between the coarse adjustment teeth and the scope body, the body tube could be raised and lowered by hand. The fine adjustment worked well and I rarely noticed the lack of a coarse adjustment knob. I could do nothing about the lack of a sub-stage condenser except to adjust the light with the concave mirror. The lenses were excellent. I used this brass microscope for almost all of the fixative investigation. In December 1971, E. Leitz repair service in Rockleigh, NJ, was able to fit the 1890 microscope with a coarse adjustment and single lens sub-stage condenser with primitive diaphragm. The latter consisted of a rotable metal disk with different sized holes detented to allow passage of light to the condenser (see Fig. 1).

By this time the reader is probably wondering, "Where is the fixative?" I had been using a number of fixatives in my work, mainly Schaudinn's, Bouin's, and Formol Acetic Alcohol (FAA). Glass slides were coated with Mayer's egg albumin and a droplet of culture was placed on the slide and allowed to almost dry. This procedure was always "hit or miss," due to the uneven evaporation of the culture droplet. The organisms

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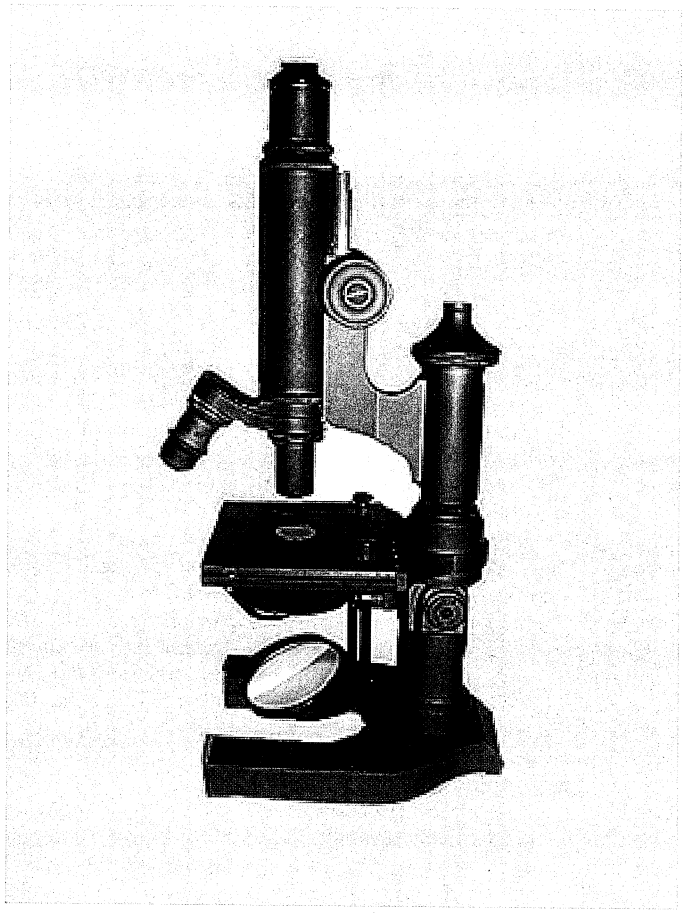

Fig. 1. Antique Leitz microscope (circa 1870) purchased by the author and used in some of the studies for the development of Nissenbaum's Fixative.

at the outer edge would die and become distorted while the central area of organisms would not be attached at all. The ring in between would contain organisms in various states of morbidity and attachment at the time the fixative was applied: this was the state of the art at that time. These fixation procedures were quite time consuming.

One evening in the lab, I accidentally knocked over and spilled FAA on slides that had droplets of *Paramecium multimicronuleatum.* I reached over and rinsed the slides with running water, in anticipation of reusing them. As I was about to wipe the slides, I noticed that they had not rinsed cleanly, and I held them under the water again with the same result. It suddenly struck me to examine these slides under the microscope, whereupon I found globs of cells, totally distorted. This was the "moment of truth!" (Pasteur had said: "In the field of observation, chance favors the prepared mind.") I immediately placed a droplet of culture on a clean slide and pipetted a few drops of FAA onto the slide. After a quick rinse in water, I observed the same phenomenon.

I discussed with Dr. Atlas what had occurred. He was quite skeptical, even after I demonstrated to him what had happened. It occurred to me that there must be a combination of reagents that would simultaneously fix and enrobe (attach) the organisms, eliminating the disadvantages of the existing fixation procedures. After a few days of experimentation, I told Dr. Atlas that I wished to set aside the work I was originally doing and try to develop a fixative using this unorthodox method. I started researching fixatives in the various texts in our library, such as Guyer (above) and *Microtomist's Vade-Mecum* (Lee 1937). I was a freshman at this time.

I decided to visit the extensive library at the Museum of Natural History in New York City. It was there that I met Dr. Libby Hyman, who helped me with my search and invited me to her laboratory. As the project progressed, I visited her often for a critique of my slides. She was dubious of the unusual methodology at first. Incidentally, we used her text, *Comparative Vertebrate Anatomy* (Hyman 1942) in our year-long sophomore course.

The project was complex. I used multiple combinations and proportions of the reagents in the known fixatives, and kept pages and pages of notes on the post-fixation morphology of the protozoa, nucleus, and degree of enrobement. The work progressed slowly, since I changed the proportions within given mixtures and wound up discarding most of them. For a period of time, I just took reagents (from acetone to zinc) off the lab shelf and tried to incorporate them into a fixative. Almost all were dead ends. During summer vacations, I kept the cultures going by taking them to the overnight camps where I worked as an American Red Cross Water Safety and Small Craft Instructor, teaching swimming, life-saving, canoeing, and sailing.

Dr. Atlas reviewed and criticized my nuclear-stained slides and their morphological naturalness, but was at a loss for suggestions, since I had progressed beyond his knowledge in this area. After about a year I began feeling that good to excellent fixation could not be achieved. At that point, the fixative contained mercuric chloride, Formalin, acetic acid, and ethyl or methyl alcohol. Mercuric chloride and alcohol are coagulant fixatives while acetic acid and Formalin are not. I found that alcohol caused shrinkage and acetic acid caused swelling. I could not seem to balance the two. Also, lurking in the background was the problem of enrobement, which was affected by the proportions of reagents used. I felt that alcohol was the prime factor in the enrobement problem. It occurred to me that I should try the higher alcohols and their isomers. Eventually, I found that tertiary butyl alcohol (TBA) fit the bill.

After a year and a half I felt that I had achieved excellent fixation and enrobement. Dr. Atlas viewed my final slides and suggested that I visit Dr. Richard P. Hall at New York University (NYU), University Heights, for his opinion.

I put a culture of *P. multimicronucleatum* in a paper bag, along with stain, a few slides, a test tube, and the four reagents in 10-ml dropping bottles, and took the bus uptown to NYU. Dr. Hall was at his desk in the lab with a binocular microscope in front of him. He motioned me to sit next to him and said, "Dr. Atlas told me you had something to show me." I briefly described my new fixation method, as I took out the reagents and placed a drop of culture on a slide. I mixed the four reagents in a test tube, picked up a pipetful and dropped it on the slide, followed by a quick rinse in water, and a nuclear stain (probably methyl green). He viewed the slide for what seemed to be a long time, took it off the stage, and held it up to the light. He then turned to me and said, "Could you do that again?" He then called in a graduate student and said to her, "I want you to see this." I was about to prepare another slide when he reached over and stopped me, saying, "This time, try mine."

He sent the graduate student for a box of slides and culture. I prepared a slide using his culture (*Tetrahymena*) and handed it to him. He looked at the slide and asked his student to view it. Dr. Hall turned to me and said, "Young man, I don't know what you've got there, but it's just what we need; publish it right away." I offered to leave the fixative with him for use in

his classes, but he refused to accept it, saying something about sending him a copy when it was published.

I returned to Dr. Atlas' lab and he apparently knew what had transpired. He suggested I write a paper describing the new method. I wrote a twenty-page article (from my extensive notes of the past year and one-half) and submitted it to Dr. Atlas with both of us as authors. A few days later, he returned the manuscript to me. The first thing I noticed was that he had crossed off his name. I asked why he did not want to co-author the paper and he said, "You did all the work. I only looked at your slides." He always corrected papers with a red china marker and when I started to thumb through the paper, I saw that he had "x'd" out every page except the last. I asked why he had done this and he said, "Don't give them the failures. Only tell them what works." I said, "But that leaves only the summary." He replied, "Then that's what you'll publish!"

I submitted the manuscript entitled "A Combined Method for the Rapid Fixation and Adhesion of Ciliates and Flagellates" to *Science* in December of 1952 and it was published July 3, 1953. I also sent the manuscript to Dr. Hall who began using the method with his classes (Hall, R.P., pers. commun.). The entire communication is reprinted below with permission from *Science* (Nissenbaum, G. 1953. A Combined Method For The Rapid Fixation and Adhesion of Ciliates and Flagellates. *Science,* **118**:31–32). Copyright (2000), American Association for the Advancement of Science.

A combined method for the rapid fixation and adhesion of ciliates and flagellates. In the preparation of protozoan slides, difficulty is often encountered in affixing the animals to the slide without distortion. The method described below eliminates the need of egg albumin and also the drying process. The whole technique takes only 15 seconds, and the animals are simultaneously fixed and attached to the slide. The method makes use of the fact that dispersal currents cause protozoa to adhere to the surface of a glass slide. Among the reagents that produce this effect are: Formalin, ethylene glycol, acetone, ether, chloroform, and the lower alcohols (methyl, ethyl, propyl, butyl, amyl) and some of their isomers. Though all these compounds cause adhesion to the slide, tertiary butyl alcohol yields the best results. Ethyl and methyl alcohols may be substituted for tertiary butyl alcohol, but they seem to cause more nuclear distortion.

A mixture of the reagents given below will affix almost all the animals in a droplet of culture. No cellular distortion occurs and cilia, cirri, cytoplasmic granules, nuclei, and neuromotor apparatus are well preserved.

Fixative:

10 vol. saturated solution of mercuric chloride
2 vol. glacial acetic acid
2 vol. commercial Formalin
5 vol. tertiary butyl alcohol C.P.
The above reagents are mixed just before using.

1. Place a large drop of culture on a slide (the droplet must be free of debris).
2. Pick up a pipetful of fixative and hold the tip about 2 cm above the slide.
3. Slowly drop the fixative directly onto the droplet of culture.
4. Once the currents have started, lower the tip of the pipet until it is in contact with the slide. Continue to expel the fixative slowly until the slide is flooded.
5. After 15 seconds drain the slide and place in 70% iodine alcohol (3–4 min).
6. Wash in 70% alcohol (3–5 min).

The slide may now be stained by any of the standard methods.

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Soon after publication, I received reprint requests and inquiries on the application of the method in special situations. I continued working with the Fixative on a new tack (e.g. developing a nuclear-stain-fixative combination). Despite intensive efforts, I was unable to obtain satisfactory fixation, enrobement, and nuclear staining in one operation. By this time, I had entered my senior year and other priorities took over (i.e. graduate school interviews, etc). With six months to graduation, I asked Dr. Atlas if another student might be interested in continuing the stain-fixation investigation. No one came forward and I closed down the project.

Epilogue. In the following paragraphs, I will attempt to trace the variegated use and modifications of the Fixative from the 1950's to the present time. I am aware of the fact that the Fixative is used in advanced biology and graduate protozoology courses. Most of the references were obtained through *Science Citation Index.* However, many were not referenced in the *Index* and were found during my own perusal of the literature. Searching the foreign literature was not easily done—papers that noted the use of the Fixative but were unobtainable were purposely omitted. For the sake of brevity, I have used the actual titles of papers in the text with author and year, and in some cases the authors' comments are also noted. They are fully referenced in the bibliography.

When I use the term "Fixative," it implies Nissenbaum's Fixative, unless otherwise noted. When the volumes of reagents in the original fixative are modified, they will follow this order: $HgCl_2$; acetic acid; 40% Formalin; tertiary butyl alcohol (TBA). Of the two final formulations, I chose the one published, though 6-2-2-4 was equally as good.

Aside from numerous reprint requests, I heard nothing further concerning the Fixative until I received a letter from Dr. Atlas in 1954. He called my attention to an abstract on the chromosome number of four varieties of *Tetrahymena* (Ray and Elliott 1954). There followed a series of papers see Ray (1956a, b). One of the advantages of the Fixative is that chromosomes are disposed in a single plane (Ray and Elliott 1954). This is especially useful in photomicrography (Ray 1956b). He had started using the Fixative in the protozoology course at Emory University (pers. commun.), as had Elliott at the University of Michigan (Elliott, A.M., pers. commun.).

Lippman (1957) described the growth inhibitory action of heparin on the Ehrlich ascites tumor in mice. She collected the tumor exudate onto slides and drained the excess, leaving a single layer of cells, which were fixed. In 1961, I found that the Fixative did not perform well with hemorrhagic pleural or pericardial fluids (Nissenbaum and Snapper 1961). Mercuric chloride ($HgCl_2$) was replaced with tetrahydrofuran (THF), which had the advantage of selectively lysing red blood cells (in hemorrhagic fluids), leaving other cellular elements intact, including white blood cells. Tetrahydrofuan also replaced the alcohol dehydration process (Haust 1958, 1959), and slides could be mounted directly from THF. The toxicity of THF is similar to ether (Malkinson and Potter 1960). I have not found any other workers using THF in the protozoological literature.

In the 1950's and 1960's a number of papers were published utilizing the Fixative in x-irradiated protozoa (Elliott and Clark

1956; Wells 1960, 1961). The cytogenetics of genomic exclusion in *Tetrahymena* was described by Allen (1967a, 1967b) and age-associated nuclear anomalies in *Tetrahymena* (Wells 1965). In the 1960's, papers started appearing from the U.S.S.R. with reference to the Fixative (Agamaliev 1967; Raikov 1960, 1962; Raikov and Volkonitin 1989; Raikov, Karadzhan, and Kaur 1989). Raikov (1962) modified the fixative slightly by doubling the quantity of tertiary butyl alcohol (TBA) (10-2-2-10). This gave better adhesion (enrobement) of marine ciliates "which were generally very difficult to attach." Agamaliev (1967) also referenced the modification. Of interest, the Fixative formula published in the *Illustrated Guide to the Protozoa* is 10:2:2:10 (Lee et al. 1985).

This modification also appeared in the stain-fixative, described by Borror (1968a), using my Fixative (12 vol) and nigrosin-formalin solution of Deroux and Faidy (1966). He named the stain-fixative "NMF" (nigrosin-mercuric chloride-formalin). Borror (1968a) had accomplished what I had been unable to do in 1953, a combined stain-fixative. To be precise, I had tried to develop a nuclear stain-fixative while "NMF" is a ciliary organelle stain-fixative. Borror (1969) later modified it for fragile or contractile ciliates. NMF stain-fixative has been used extensively in ciliate systematics. Parker and Giese (1966) used the same modified proportions (5-1-1-5) in their study of nuclear activity during regeneration in *Blepharisma.* They noted "excellent preservation and adhesion of the cells." Additionally, to prevent movement of the cells over the slide, a "ring" of fixative was pipetted rapidly around the culture drop. To achieve uniform adhesion, the slide was held at a 45° angle and flushed with fixative to drain off completely all culture medium. Kennedy (1965) used the Fixative, along with others, to study the complex morphology of the fibrillar system in *Blepharisma undulans.*

In studying amoebae, the Fixative has been used in a wide array of subjects including description of new species (Page, 1967a, 1967c, 1971b). Page (1967b) used it to redefine the genus *Acanthamoeba,* a freshwater amoeba that had been reported to cause human fatalities by attacking the brain and other organs (Culbertson 1981; Culbertson and Harper 1980). Carter (1970), at the Adelaide Children's Hospital, South Australia, used the Fixative for fixation of cysts in his study of experimental pathological changes induced by *Naegleria* sp. in primary amoebic meningoencephalitis. Shumaker et al. (1971) isolated *Naegleria* from the nasal swabs of a healthy individual. *Limax* amoebae (among other ciliates and flagellates) were recovered from an indoor physiotherapy pool (therapie-schwimmbad) in a German hospital (Michel and Snyder 1980). The Fixative was also used in a survey of pathogenic, free-living amoebae in a swimming pool in Mexico (Rivera et al. 1983). Rivera et al. (1984) also identified pathogenic and free-living protozoa from the nasopharyngeal and oral regions of dental patients using the Fixative. Culbertson (1981) employed Page's modification (Page 1967c) with an indirect immunofluorescence method, using formalinized, stained protein A staphylococci. His procedure compared well with the direct immunofluorescence method of identification (Culbertson and Harper 1980). Culbertson (1981), a clinical pathologist, wrote an extensive review of amebic meningoencephalitis, along with laboratory findings and methods. He noted the best preparations were produced with Nissenbaum's or Papanicolaou's Fixatives, which "provided for clearer distinction of the amoebae from tissue cells."

The method has also been used extensively in marine biology (Raikov 1960; Raikov, Karadzhan, and Kaur 1989), excluding descriptions of new species. Investigations ranged from the Atlantic Ocean (Nerad et al. 1995) to the White Sea (Raikov 1962), Caspian Sea (Agamaliev 1967) and Japan Sea (Raikov and Volkonitin 1989), notwithstanding rivers and draining sewage plants (Sawyer, Nerad, and Munson 1992), to ciliates found in tidal flats (Epstein, Burkovsky, and Shiaris 1992), tidal pools (Borror 1962), and among sand grains (Epstein et al. 1992). The description of new species (Beers and Sherwood 1966; Borror 1966; Page 1967a1967c, 1972, 1979, ; Page and Kalinina 1984; Napolitano, Wall, and Ganz 1970; Nerad et al. 1995; Raikov 1962; Raikov and Volkonitin 1989; Read et al. 1983; Sawyer, Nerad, and Munson 1992; Sawyer et al. 1998; Siemensma and Page 1986; Willumsen 1982) and taxonomic criteria (Borror 1968b; 1971a; Carter 1972; Frederick 1972; Page 1968; 1974a; Wells 1960; Willumsen, Siemensma, and Suhr-Jessen 1987) have been published utilizing the Fixative.

The Fixative was used in studies of conjugation in the ciliates *Paramecium polycaryum* (Diller 1958), *Tetrahymena thermophilia* (Ron and Suhr-Jessen 1981; Weiske-Benner and Eckert 1985) and *Aspidisca costata* (Diller 1975), and in studying mitosis in *Gonium pectorale* (Shyam and Sarma 1975). In regard to algal flagellates, Shyam (1978) described a double fixation method using the Fixative and Carnoy's Fluid, followed by aceto-carmine stain. He noted that the procedure is highly advantageous in securing a) rapid and firm adhesion of the flagellates to the slide, b) better contrast in chromosome staining, and c) effective spreading of chromosomes. Page and Willumsen (1980) reported observations to revise the description of *Gocevia placopus,* which over many years had been variously described by earlier authors.

Old and Darbyshire (1978) used the fixative in their study of soil fungi as food for giant amoebae. Spore perforation by amoebae was initiated by an annular depression in the spore wall, which was eventually breached and pseudopodia penetrated the spore lumen. The cell contents and septa were completely digested in four to five hours.

The fixative has been employed in genetic studies: for example, the role played by the nuclear envelope in chromosome segregation in *Oxyrrhis marina* (Cachon, Cachon, and Salvano 1979), changes in the DNA content of differentiating macronuclei of the ciliate *Loxodes magnus* (Bobyleva, Kudrjavtsev, and Raikov 1980), and genetic instability in the mating type system of *Tetrahymena pigmentosa* (Simon and Orias 1987).

Wee (1983) published a paper on specimen collection and preparation for critical light microscope examination of Synuraceae (Chrysophyceae), in which a major portion was devoted to the use of the fixative in identification of Synuraceae compared to the benchmark method of electron microscopy. His method, which used the fixative, yielded information from images of both scales and cells. He noted that the slide need not be flooded with fixative as originally published—only one drop of fixative on the sample would avoid losing specimens. Although not in my original paper, I found that once the dispersion currents started, one could "chase" them and drop fixative directly onto the currents. I had mentioned this in the original paper, but it had not appeared in the short summary in *Science.* Also left out of the original paper was the rapid removal of iodine with a five-second dip in 1% sodium thiosulfate solution, ($Na_2S_2O_3$).

Jamieson and Anderson (1972) described a simple method for studying nuclear division in free-living soil amoebae. They inoculated amoebae on agar plates, cut blocks from selected areas of heavy growth, and gently pressed clean glass slides onto the block surface, thus transmitting the organisms to the slide, which was quickly reversed and flooded with fixative. Kovalchuk (1980) described a quick method of making permanent preparations of ciliates in field conditions using the Fixative.

Following initial fixation, some investigators employ a second fixative for a few minutes to hours, such as $HgCl_2$/acetic acid (19:1 or 20:1) (Page 1974a, 1974b, 1979, 1983; Page and Kalinina 1984; Page and Willumsen 1980). Ray (1956a) employed a double fixation with Nissenbaum's and Carnoy's Fixatives, followed by hydrolysis in normal HCl, before staining in aceto-carmine, which gave the most satisfactory preparations for detailed chromosome study.

Cather (1958) modified the Fixative for fixation and staining the chromosomes in eggs of invertebrates. These are yolky in many marine invertebrates and are difficult to handle and stain, especially when a detailed study of the nucleus is desired. Barrett (1968) published methods for demonstrating cells in calcareous and other sponges (either prefixed or living). The fixative was used to affix the sponge cells to the slide.

Various stains have been used following fixation. The most frequent appear to be aceto-carmine (Ray 1956a, 1956b), acid fuchsin (Napolitano, Wall, and Ganz 1970), Feulgen reaction (Chen, Luo, and Cao 1982; Ron and Suhr-Jessen 1981; Wee 1983), Giemsa (Culbertson 1981), hematoxylin (Ron and Suhr-Jessen 1981; Willumsen 1982), Kernechtrot (Willumsen, Siemensma, and Suhr-Jessen 1987), methyl green and pyronin (Kovalchuk 1980), NMF (Borror 1969), and protargol method (Borror 1962, 1966, 1968b; Read et al. 1983; Shyam and Sarma 1975). With regard to the Feulgen reaction, Golikova et al. (1980) studied the intensity of the reaction in macro- and micronuclei of *Paramecium bursaria* under various conditions of hydrolysis and age of the organism. They found the most intense staining depended on the age of the clones: younger clones stained more intensely and rapidly than older ones. They ascribed this to a stronger binding of proteins to the DNA, and an inhibition of the DNA degradation during hydrolysis.

Postscript. In the foregoing, I have touched upon the wide variety of applications (and modifications) the Fixative has undergone since being published, forty-seven years ago. The unprecedented method of dropping a clear liquid solution on a drop of culture material and instantaneously fixing and enrobing the organisms to the slide in a natural state was inconceivable, even to the biologists and protozoologists to whom I demonstrated it. In retrospect, I have come to the realization that I was dealt with honestly and fairly, though skeptically by my own professor Meyer Atlas and leading biologists, Drs. Richard P. Hall and Henrietta Hyman. I was fortunate that Dr. Atlas did not reject out of hand my enthusiastic proposal to develop a new fixative method after perusing my original slides, fixed with formol-acetic-alcohol (FAA), on which he saw only globs of unidentifiable protoplasm. Lastly, I am indebted to Dr. John O. Corliss, "Father of Ciliate Systematics," for graciously including my method in his treatise, *The Ciliated Protozoa* (1961, p. 183), under Technical Advances of the Greatest Importance During the Last Century, as one of the "pioneering cytological techniques."

Following graduation from college, I entered medical school at the State University of New York. There followed an internship and residency in Internal Medicine. In 1961 I was awarded an NIH Research Fellowship in Gastroenterology from the National Cancer Institute.

In 1961 Dr. I. Snapper and I published "A New Rapid Method For Preparation Of Exfoliated Cells Obtained From Body Fluids," (Nissenbaum and Snapper 1961). During my years as a Fellow in Gastroenterology, I developed a cine-photographic system for gastrointestinal endoscopy (Nissenbaum, DiBianco, and Groisser 1963) and patented a device, which was manufactured commercially, for the localization of gastrointestinal bleeding (Nissenbaum et al. 1965). In 1963, I entered private practice in Internal Medicine and Gastroenterology and was appointed Assistant Clinical Professor of Medicine at the University of Medicine and Dentistry of New Jersey. I am still in private practice and also serve as an attending physician at the Jersey City Medical Center, where I teach medical students, residents, and fellows (Fig. 2).

Fig. 2. Gerald Nissenbaum. Photograph taken in 1996.

If there is anything to be learned from my experience, it is this: young investigators with creative ideas deserve encouragement to pursue them. After all, it is the latter who will enthusiastically put forth the effort, guided by their mentors. They should be allowed to "run free" and even "break out a spinnaker" rather than sail "close hauled." To quote the motto over the doors of the State University of New York—"Let each become all that he is capable of being."

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