

# A Short History of the Initial Application of Anti-5-BrdU to the Detection and Measurement of S Phase

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Because this article is a bit of history rather than a scientific paper, we will depart from the formality of a journal article. This article has two purposes. The first is to give credit to two deceased individuals, Albert Castro, M.D., and Howard Gratzner, Ph.D. (1). Castro was the key person who was able to produce a polyclonal antibody that was specific for bromodeoxyuridine (BrdU). For 10 years, Gratzner took the lead in converting an idea into a functional assay that affected DNA analysis and led to the development of many other assays with similar methodologies. The second is to inform young scientists that (a) inventions or creations have utility in fields other than the purpose for which they were created; (b) it is possible to do useful work in an institution that certainly would not have been classified as one of the best; and (c) the presence of talented people who are willing to collaborate is invaluable.

Scientifically, Gratzner is best known for the development of the first monoclonal antibody (mAb) to BrdU and iododeoxyuridine. This work was the culmination of previous efforts with Robert Leif to develop polyclonal antibodies, which detected BrdU with variable specificity. Collaborations with scientists at the Lawrence Livermore Laboratory encouraged the development of a widely used flow cytometric test for the simultaneous measurements of DNA and BrdU. The use of mAbs to halogenated pyrimidines was a significant scientific advance and currently is an integral tool for the analysis by flow and digital microscopy of the cell cycle.

On a personal level, Gratzner's enthusiasm for science and his intelligence, curiosity, loyal interactions with fellow collaborators, and friendship are sorely missed by all who knew him. He was genuinely a warm and nice person who was liked by almost everyone with whom he came in contact (1).

Castro was a jovial man who interacted well with his collaborators. He was generous and very knowledgeable. He ran an immunodiagnosics laboratory at the universities of Oregon and Miami and was an expert in the production of antibodies in animals. His warm smile, generous giving of his knowledge, common sense, enthusiasm for scientific research, and ability to get the job done were essential to this project.

Because this is a story of events that happened approximately 30 years ago and the existing records are limited, the accuracy of this article is limited by our memory and those of our colleagues who have kindly helped with this project. Of course, this is our version of the events.

Cell division and DNA replication are fundamental to biology and specifically to the biology of cancer. This article describes how a simple procedure for the detection and quantitation of DNA synthesis was developed. The precise determination of when S phase occurs in the cell cycle is of use to maximize the selective killing of tumor cells with cycle-specific chemotherapeutic drugs. Of greater significance, studies of the control of the cell cycle, including its detour into apoptosis, can provide extremely useful insights into the creation of new therapeutic regimens.

The original process to detect S-phase cells included the use of tritiated thymidine with autoradiography to measure the labeling index (percentage of S-phase cells) and/or the fraction of labeled mitotic cells. Before and at the infancy of flow cytometry, it was believed that the quantitative DNA analysis of normal and neoplastic cells might provide an objective marker for the diagnosis of neoplasia. These measurements were performed with a microscope on Feulgen-stained cells. In a normal population, it was established that there were two predominant peaks in the DNA distribution, with a ratio of 1:2 in DNA content, and that cells that were synthesizing DNA (S phase) were scattered in between, with a relative DNA content between 1 and 2. The detection of tritiated thy-

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In memoriam: Albert Castro and Howard Gratzner, for their pioneering studies in the application of anti-5-BrdU for the detection and measurement of S phase of the cell cycle.

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midine incorporation by autoradiography was the technique for marking cells in S phase. The immunochemical measurement of S phase greatly facilitated cell cycle analysis of samples that were a mixture of normal and aneuploid cells. This communication describes the history of the early development of this technique.

Early flow cytometers had the capacity to detect one fluorescence parameter in addition to light scatter parameters from cells or nuclei. Van Dilla et al. (2) initially used the fluorescent Feulgen reaction to quantitate DNA and observed the G1 and G2 peaks of the cell cycle. Subsequently, Crissman et al. (3) and Krishan (4) replaced this acid-based technique by the use of DNA-binding fluorochromes. The capacity of flow fluorometry to precisely measure the DNA content of a statistically significant number of cells greatly improved cell cycle analyses. However, flow cytometers are unsuited for conventional autoradiographic measurements including that of tritiated thymidine incorporation. This article describes a fluorescence method, the use of a fluorescent antibody, to detect the incorporation of BrdU into DNA.

There are three major technologic requirements for the clinical flow cytometric measurement of 5-BrdU incorporation into cellular DNA: (a) 5-BrdU must be made available to and incorporated by the cells; (b) single-cell measurements by flow cytometry have always required producing a monodisperse cell suspension; and (c) there has to be a means to detect the incorporation. The incorporation of 5-BrdU can be accomplished by direct injection of the labeled nucleotide into the patient, labeling of tissue fragments or slices, or adding 5-BrdU to single cells in a culture medium. Direct injection of a labeled nucleotide into humans is potentially harmful and requires a large amount of an expensive compound. Because tissue fragments or slices no longer have molecules delivered by the circulatory system, these molecules, including the labeled nucleotide, must be delivered by diffusion. The limitations on diffusion imposed by the tissue architecture will result in spatial differences in concentration. Another possible solution is to dissociate the tissue slices or fragments into single cells before exposure to the labeled nucleotide. In any event, after *in vivo* and before or after *in vitro* incorporation, monodisperse cell preparations have to be created. The simplest solution is to produce nuclei; unfortunately, the loss of the cytoplasm very often precludes the ability to connect the individual DNA measurements with a specific type of cell. Dissociation of tissue slices or fragments was and remains problematic.

Although in hindsight it seems logical to detect S phase by the fluorescence of antibody reacting with BrdU that had been incorporated into cells, it should be emphasized that in the mid-1970s this was far from guaranteed to work. In the mid-1970s there was limited application of antiserum against biological molecules, and the creation of mAbs had not yet occurred. The technical difficulties of reproducibly producing a polyclonal antibody specific for 5-BrdU were significant.

## HISTORY

### Polyclonal Anti-5-BrdU

Although the introduction of this article has provided a rational and orderly view of the development of anti-5-BrdU, this was not the case. The application of anti-5-BrdU for the detection and measurement of S phase was the result of necessity, not of foresight or wisdom. In essence, one invention by Leif, centrifugal cytology, forced his invention of the use of anti-5-BrdU for measuring S phase. Centrifugal cytology is a process that centrifuges cells onto a slide and then fixes the wet cells under the action of centrifugal force. This process is based on G. N. Papanicolaou's great technical contribution to cytology, wet fixation (5). The surface tension forces produced by air drying from water are sufficient to disrupt and severely distort the morphology of cells, in particular active cells.

Because linear gradient buoyant density centrifugation of human erythrocytes (6), guinea pig bone marrow (7), and other cells (8,9) had indicated that buoyant density is related to cell age, it became of interest to perform tritiated thymidine labeling (10). Conventional liquid emulsion technique required air drying from the emulsion, which distorted the cellular morphology. Marilyn Cayer, one of Leif's longest term collaborators, provided a solution to this problem. Fortunately, she was and is a very talented electron microscopist. She embedded cells that were bound to the microscopic slide, sectioned them in the direction parallel to the microscopic slide surface, and then covered the sections with liquid emulsion. This resulted in a preparation that allowed reliable counting of silver grains. Unfortunately, the technique was somewhat arduous and time consuming and could be performed only by a few superbly talented individuals. Frankly, Leif certainly was not one of them. He therefore started a dialog with Gratzner concerning the use of 5-BrdU. Gratzner was the most knowledgeable scientist concerning molecular biology at the Papanicolaou Cancer Research Institute (PCRI) and was willing to spend time discussing the problem. From Leif's days as a graduate student in Jerome Vinograd's laboratory at Caltech, he had known that 5-BrdU could be incorporated into DNA. The incorporation of 5-BrdU by cells increased the buoyant density of their DNA and thus permitted the separation by cesium chloride density gradient equilibrium centrifugation of newly synthesized DNA from unlabeled DNA.

The anti-5-BrdU story begins one evening (approximately in 1972) while Leif was sitting at home reading a journal. It dawned on him that all of his previous wild ideas were the wrong approach. Why not make an antibody against 5-BrdU and use it to detect the incorporated nucleotide? Leif excitedly called Gratzner on the phone and asked his opinion. Gratzner said it made sense. For Gratzner and Leif, this approach posed a significant problem. Leif had never made an antibody; and although it is not known if Gratzner had, Leif remembers that Gratzner had never done so starting with a hapten. Therefore, Gratzner and Leif went to see Castro, who had been hired by the University of Miami to set up an immunodiagnostic

laboratory. For Castro, this was not black magic but a known, reproducible procedure. Castro, in addition to being an expert on antibody techniques, was extremely practical, intelligent, kind, and readily shared his knowledge. Castro was a very large man. He had been brought up in Central America, and he not only looked like Santa Claus with his size and weight but became the Santa Claus of the project. Gratzner and Leif soon learned that Erlanger and Beiser had already made an antibody against 5-BrdU (11). Castro soon had a good affinity-purified polyclonal antibody, and Gratzner, with help from one of Leif's technicians, Diane Ingram, characterized it initially by double diffusion and micro-complement fixation.

Gratzner and Leif were very pleasantly surprised and Castro was pleased but not surprised that they were able to report (12) double-diffusion studies demonstrating that antisera forms a precipitin band with 5-BrdU-conjugated bovine serum albumin (BSA) and not with BSA or the thymidine-BSA conjugate. The blocking of the formation of the bands by the presence of 5-BrdU in the agarose provided reassuring confirmatory evidence. The 5-BrdU-containing DNA was six times more effective than the original 5-BrdU-BSA conjugate in fixing complement. Some success in this experiment was derived from colleagues at the University of Miami Department of Microbiology who worked in DNA chemistry. Leif suspects that Sheldon Greer, who had done extensive studies on halogenated bases (13), or one of his colleagues in the Department of Microbiology at the University of Miami was the one who told us the trick of maximizing the incorporation of 5-BrdU into DNA by adding 5-fluorodeoxyuridine to block thymidine formation. Four papers coauthored by Gratzner and Leif have been published and have been posted on Leif's Web site (14).

As was the custom at that time, an indirect immunofluorescence procedure using fluorescein-labeled goat anti-rabbit immunoglobulin G (IgG) was used to demonstrate specificity. The researchers at the PCRI collaborated as a team, with each taking different responsibilities. Gratzner was the biologist who had the joy of discovering that BrdU labeling clearly shows a sister-strand chromatid exchange and focusing his efforts on biological applications. Leif was acting as the biophysical chemist whose role was to suggest the mode of DNA denaturation, which was based on what he had learned from others including his mentor, Jerome Vinograd, at Caltech. Denaturing of DNA was a key step because the antibody could recognize only 5-BrdU present in single-stranded DNA; and unless the DNA was presented to the antibody in a correct configuration, no activity would be detected. Gratzner and Leif evaluated the experimental data including the microscopic preparations to be sure that their morphologic conclusions were consistent. The end result of this work is best described by an excerpt from the first paragraph of the 1975 paper (12):

This report describes the development of an immunofluorescent method for identifying DNA synthesis in single cells, utilizing antibody specific for 5-bromo-2-

deoxyuridine (BUdR). The technique enables rapid processing of slides for the measurement of cell kinetics, permitting analysis in a matter of hours. The technique appears to be an alternative to autoradiography and has applications for flow fluorometric quantitation of de novo DNA in single cells or isolated nuclei.

Sufficient data had been obtained, so funding proposals were submitted to the National Cancer Institute (NCI) and the National Institutes of Health (NIH). Human relations and financial considerations always play a part in scientific research including those at the PCRI. Julius Shultz, the director of the PCRI, operated the PCRI on soft money, which probably was the policy of the board of directors. Therefore, it was essential for all the investigators employed there to generate their own research support including the overhead necessary to run the institute. To stay in Shultz's good graces, it was necessary to make good scientific presentations to on-site visits by NIH study sections and the PCRI's scientific advisory board. Unfortunately, Gratzner's presentations lacked charisma and stage presence. Parenthetically, these personality traits have nothing to do with the capacity to do good scientific research. Thus, in the eyes of the director, Gratzner did not fit into the category of successful scientists. In fact, Gratzner was in serious danger of being fired unless his research was funded and peer review demonstrated scientific significance. An NCI contract proposal, "Markers for Instrumental Evaluation of Cells of the Female Reproductive Tract," was funded, thereby permitting Gratzner to continue his employment and research at the PCRI. Gratzner was the co-investigator for the 5-BrdU component, which was a significant part of the NCI contract. Gratzner included input from Leif's group: Marilyn Cayer, the laboratory technicians, the graduate students, Richard Thomas, and Jerry Thornthwaite. Leif's role as principal investigator included writing part of and integrating the rest of the content of the contract reports and making 50 xerographic copies to send to the NIH.

A second contract was submitted to the Breast Cancer Section of the NCI to fund the development of this 5-BrdU assay to quantify S-phase cells by flow or image. The application in this case was to enable the clinician to use cell cycle information to improve the use of breast cancer chemotherapy. This contract included the funding for an expensive fluorescence microscope to evaluate the 5-BrdU antibodies. This microscope was essential for this and other projects at the PCRI. A junior scientist, Robert Zucker, was a co-investigator on the contract. Zucker had been a graduate student in the same laboratory at University of California at Los Angeles, where Leif had a postdoctorate. Zucker had just returned from a postdoctorate at the Max Planck Institute in Munich, Germany and was funded for his first year at the PCRI on soft money from Leif's and Bruce Cameron's (another PCRI investigator who was working on sickle cell anemia) grants.

The site visit team reviewed the proposal, with Gratzner as the principal investigator and Zucker and Leif

as co-investigators. Although the committee was extremely impressed with the quality of the proposal, they were dissatisfied with the quality of Gratzner's presentation describing the proposal. The NCI committee believed that Gratzner did not have the potential to effectively lead this project. They offered to fund the proposal to the PCRI, but only if Zucker was the principal investigator and Gratzner was the co-investigator. The PCRI director, Julius Schultz, was not about to turn down a lucrative contract with the NCI and agreed to reverse the roles of his two investigators. Although this is an unusual practice, the grant can be awarded to the institute and the director of the institute can change the principal investigator. Gratzner was a hard worker who could not express himself with the confidence necessary to convince people that he could get the job done. He was basically a pessimist; and society including science really values and rewards optimistic individuals. In fact, Gratzner's lack of confidence was a credit to his scientific integrity. Part of the contract proposal was based on prior work by Leif's group on the dissociation of bovine pituitary (15), which in hindsight was much easier than dissociating tumors (16).

In contrast, Zucker had a good curriculum vitae and a good track record for someone 30 years old, and he took on the responsibility of being a principal investigator for dissociating breast cancer cells with the vision of eventually measuring their characteristics with a flow cytometer and a microscope. However, his role was diluted as he wrote three other grants on returning from his postdoctorate and found himself now managing four funded grants, including the NCI contract. Leif also helped to the best of his ability to keep this 5-BrdU project focused to achieve the goals. However, Leif had responsibilities of his own, including building a flow cytometer that had the capacity to do simultaneous multiparameter analysis in addition to work on his own contract on cell markers.

In all, Gratzner was really the main person who performed the day-to-day activities and kept his nose to the project and ran the necessary experiments to achieve results. He accepted the responsibility and focused on this area of research (5-BrdU antibody detection of S-phase cells) for many years. The scientific papers based on this work at the PCRI (12,17-19) provided the scientific community with a simple reliable method to obtain cell cycle data. Hence, although charisma, stage presence, and image are important to obtain the necessary funding, it is substance, hard work, and the ability to do the best possible job with one's talents that are essential qualities to achieve the goals of the project.

A subsequent paper (17) describing the recent progress with the polyclonal antibody to 5-BrdU was published in the Proceedings of the Fourth Engineering Foundation Conference on Automatic Cytology, which was the precursor of the ISAC conferences. A comparison was made of the labeling index determined by immunoperoxidase anti-5-BrdU and autoradiography. The results showed a surprisingly good agreement. The agreement with total incorporation of  $^3\text{H}$ -BrdU as measured by liquid scintillation counting with the cellular-based measurements was

mediocre at best and confirmed Leif's bias in favor of cellular measurements. Gratzner developed the chromosome banding studies. Obtaining a good picture of 5-BrdU pulsed labeled cells was an obvious necessity. Because Gratzner was far more knowledgeable than Leif about tissue culture, Gratzner likely developed the incorporation conditions. Gratzner also probably was responsible for the 5-thiouridine studies. Alan Pollack had joined Leif's group and later was associated with Gratzner doing 5-BrdU research. He performed many useful 5-BrdU experiments, and these gave a preview of his myriad of talents. He proceeded to acquire a Ph.D. and an M.D. He is now a clinical department chair.

The next paper (18) was an article produced at the request of George Wied (International Academy of Cytology) in which Leif and his collaborators described many of the present and future markers for automated Pap smears and other exfoliative cytology specimens. Parenthetically, articles, including one by Leif (20) in a 1970 volume edited by Wied and Bahr, documented the early history of analytical cytology. At that time, Leif was under the naive opinion that flow cytometry was the modality of choice for this purpose. However, later work demonstrated an inability to dissociate cervical vaginal cells (21) into the single-cell suspension necessary for their flow cytometric identification. Normally, Leif, being the director of his laboratory, was not a first author on these papers. However, because the article on the present and future markers integrated the work of his group and he did the plurality of the writing, Leif took the liberty of being first author.

The section "Possible Specific Molecular Biologic Fluorescence Descriptors of Neoplasms" stated the possibility of the use of DNA synthesis as one of the indicators of neoplasia and how the signals produced by the replication of the bacteria present in the samples could be eliminated by the use of multiparameter analysis. The tedious and difficult to automate counting of silver grains with a microscope could now be replaced by an immunofluorescence flow cytometric method using anti-5-BrdU. Gratzner and Leif also speculated on the possible use of antibodies against the single-stranded DNA regions that exist in S phase and described the state of their work with anti-5-BrdU. They first pointed out the obvious advantage of the rapid availability of the results versus tritiated thymidine autoradiography and then showed an image of a fluorescent nucleus that had been pulsed for 30 min with BrdU. They stated that, "An immediate application of anti-5-BrdU antibody has been for the analysis of cell kinetics." They had to admit that, in the case of cervical specimens, the present procedures would have to be changed to permit an incubation of a liquid specimen for 30 min before fixation. Although this is probably not acceptable to the present practitioners for Pap smear samples, it was reasonable for monolayer Pap slides and fine-needle biopsy preparations, both of which could already be prepared by centrifugal cytology. Because this chapter described a comprehensive molecular approach to the cytochemical classification of cervical vaginal cells,

it also included a description of the potential use of rare earth compounds as luminescent tags and the use of centrifugal cytology for the immunofluorescence detection of viral markers.

The NCI contract on markers that helped support Gratzner was cut and, hence, stopped his work on anti-5-BrdU; the contract was transferred to the University of Miami and subsequently terminated. The PCRI had tried to maintain its own identity by discouraging scientific cooperation between itself and the newly created Comprehensive Cancer Center at the University of Miami. Many of the senior researchers at the PCRI including Leif strongly disagreed with this policy and believed that it would imperil their academic appointments and collaborations at the University of Miami and sour the PCRI's relations with the NCI, which could result in diminished funding. Because neither Leif nor Zucker had any extra grant funds to support Gratzner, the PCRI director used the principle of soft money funding as the reason to fire Gratzner. Soft money has its benefits; but it has the serious limitation of being ephemeral. Fortunately, Marylou Ingram had just started the Institute for Cell Analysis at the University of Miami with Wallace Coulter's generous support. She gladly supported Gratzner as a member of the Department of Medicine, which allowed him to continue the 5-BrdU projects.

Parenthetically, Gratzner stated that he gave up a tenured full professorship at the University of South Florida in Tampa to please his wife and took a position on soft money at the PCRI in Miami. The greater availability of cultural events also played a part in his decision to move to Miami. During the early 1970s, it appeared that the initiation of the national war on cancer would provide a good possibility of obtaining funding for meritorious grants. The 1960s was a very good time for scientific funding; in some years, almost 100% of approved grants were funded. Why would the 1970s be any different? It appeared to Gratzner that soft money at a small institute was very achievable and would be not a major obstacle in his scientific career. However, as discussed above and later on in this chronicle of events, this was not the case and was a big mistake in his judgment.

Just after Gratzner left the PCRI, Gratzner and Leif did the work to produce the *Cytometry* paper (19) demonstrating the use of anti-5-BrdU for flow. Both researchers had realized that their S-phase technique had to be combined with a quantitative measurement of DNA. From Leif's first course in organic chemistry, he was well acquainted with the formation of hydrazones by reaction of a hydrazine with an aldehyde. Therefore, he proposed using a modified Feulgen reaction in which the acid treatment to expose the pyrimidines would be used to form apurinic acid or, he hoped, the hydrazines would displace the purines from the deoxyribosides. "They [the cells] were washed in H<sub>2</sub>O and suspended in a solution of 0.2% dansyl hydrazine (Sigma Corp, St. Louis, MO) in NHCl [an abbreviation for 1 normal HCl] and agitated for 30 min. Subsequent to this step, which is a Feulgen-type reaction, and which results in depurination of adenines and permits

antibody binding to the incorporated BrdUrd" (19). After this reaction, the nuclei fluoresced blue. Unfortunately, the mercury arc illumination of the Phywe ICP22 Gratzner used was incapable of two-parameter measurement. For one quick measurement, Awtar Krishan kindly let us use his microphotometer and we obtained something that looked like a DNA distribution. The cell preparation procedure was complex because it was necessary to denature the DNA to expose single-stranded DNA that could react with anti-5-BrdU.

Recently, the antibody-based detection of 5-BrdU has been drastically improved by newer techniques based on the same anti-5-BrdU concept, which did not require denaturation of DNA. These have been reviewed in the latest edition of Shapiro's book (23). The technique of labeling strand breaks induced by photolysis developed by Darzynkiewicz's laboratory (24) was preferred because it permits the maintenance of antigenicity. The direct binding of anti-5-BrdU to the 5-BrdU present in photo-induced strand breaks also has been reported by Hammers et al. (25).

The first two paragraphs of the discussion section of the *Cytometry* article by Gratzner and Leif (19) provided a preview of coming attractions:

A technique for the measurement of cell proliferation by an FCM [flow cytometry] immunofluorescence method has been demonstrated. The method permits relatively small amounts of BrdUrd to be detected in DNA. With the human lymphoblast cell line used in this report, pulses of 30 min (or possibly shorter) can be detected. This provides the FCM counterpart of autoradiography. An important feature of this technique is the correlation between the amount of BrdUrd incorporated into DNA, as measured by radioisotopic methods and the fluorescence intensity produced by specific antibody binding to BrdUrd. This observation suggests that the amount of DNA synthesized in individual cells can be measured, permitting, for example, analysis of drug effect on DNA elongation and/ or mutation, as well as other applications.

One advantage of an FCM technique, analogous to the <sup>3</sup>H-thymidine autoradiographic procedure as opposed to the conventional FCM DNA histogram method, is that it enables cohorts of cells to be followed independently of their DNA content as they traverse the cell cycle [26]. If a second parameter, such as DNA content or cell size, were available it would be possible to monitor the accumulation of cells entering specific compartments for the measurement of cell cycle parameters [27].

The flow cytometry anti-5-BrdU-labeled control (non-BrdU) cells exhibited nonspecific cytoplasmic fluorescence, which was proportional to the low-angle light scatter signal (cell size). Fortunately, the monumental contribution of the creation of a method to manufacture

mAbs by Kohler and Milstein (28) eliminated this problem.

At that time, Leif's renewal grant for the creation of the automated multiparameter for cells (AMAC) did not receive funding. Leif had spent most of the previous \$500,000 in grant funding on instrumentation including development of the first instrument software that ran on a minicomputer; however, this funding was sufficient only to produce a prototype. He did not have a commercial, reliable instrument to use for flow cytometric experiments. Wallace Coulter kindly supported Leif's research at the PCRI. Unlike the present, in the 1970s, corporate sponsorship was frowned upon by some academic individuals, particularly the director of the PCRI. Because the director was very uncomfortable with PCRI's corporate relationship with the Coulter Corporation, including the provision of minimal overhead and Leif's continued collaboration with the Miami Comprehensive Cancer Center, after about a year, Leif also was fired.

In 1985, the PCRI ceased to exist. The dilapidated, two-story PCRI was demolished and replaced by a modern, 12-story cancer hospital affiliated with the University of Miami. The University of Miami absorbed scientists with current grant support. Zucker, who was completely funded for the 10 previous years, found himself without grant support in early 1984. He left Miami in early 1985 and began to work for the Environmental Protection Agency as a scientist directing a core flow cytometry facility.

The PCRI had the great advantage of having a group of extremely able, dedicated, hardworking, and well-trained (G. N. Papanicolaou and E. Ayre) cytotechnologists who made very significant contributions to centrifugal cytology and the rest of Leif's work on cytologic preparation techniques. It also permitted Gratzner, Leif, and Zucker to interact with some gifted scientists such as Fazal Ahmad, Zbynek Brada, Bruce Cameron, Marilyn Cayer, Elli Kohen, and David Smith.

Wallace Coulter hired Leif to continue developing his multiparameter transducers (29) and to conduct biological studies with Wallace Coulter's instrumentation that measured direct current and radiofrequency impedance (30). The Coulter Corporation's five-part differential analysis technology was an outgrowth of this work. Frankly, the actual cost of developing clinical instrumentation is so great that it was naive of Leif to believe that he could achieve anything beyond the completion of a laboratory prototype at a research laboratory. Take-home lesson: sometimes naiveté helps. Another instrument based on the early AMAC eventually was developed and commercialized at NPE Systems by Richard Thomas who was one of Leif's energetic graduate students at the PCRI.

#### **Monoclonal Anti-5-BrdU**

After the study by Kohler and Milstein (28), it was obvious that a mAb against 5-BrdU should be created. Gratzner was now employed at the Institute for Cell Analysis, located on the University of Miami Medical School campus and financed by Coulter Corporation. Gratzner

hired a technician, Jeanne Stein, who had acquired training in cell fusion, which is an essential part of the technique developed by Kohler and Milstein for preparing mouse mAbs directed to specific haptens. Her assignment was to produce a mouse mAb against 5-BrdU with greater specificity and affinity than that of the polyclonal antibody.

Mice were injected with BSA-iodouridine conjugate according to the procedure of Erlanger and Beiser: periodate cleavage of the ribose ring of the iodouridine analog of iododeoxyuridine followed by borohydride reduction for a Schiff base condensation with the lysine residues of BSA. In accordance with the method of Kohler and Milstein, spleen cells were harvested from mice showing an immune serum reaction and fused with nonsecreting mouse myeloma cells. The resultant immortalized cells, hybridomas, were diluted and cultured in microwells, and the culture supernatants were tested for antibody secretion by micro enzyme-linked immunosorbent assay. The very first fusion yielded several hybridoma clones secreting immunoglobulins fixed by immobilized BSA-iodouridine in micro enzyme-linked immunosorbent assay wells. An IgG1-secreting clone was selected and subcloned to yield a stable species.

By micro enzyme-linked immunoassay, the anti-5-BrdU mAb was shown to have high affinity for BrdU-labeled DNA, and a quantitative reaction with the hapten was obtained at sufficiently high mAb titers. Further, microscopic preparations of whole cells pretreated with BrdU and then stained with anti-5-BrdU mAb and fluorescein-conjugated bovine anti-mouse IgG antibody showed intense nuclear fluorescence, indicating the feasibility of the application of this mAb to cytology and to the measurement of the kinetics of DNA biosynthesis by flow cytometry (31). These expectations were realized by Gratzner and collaborators in further studies of DNA replication by flow cytometric analysis (32) and fluorescence microscopy of dipteran larval polytene chromosomes (33). A collection of early papers (34) indicates the initial interest and rapid development of this technology.

An interesting extension of the work on mouse anti-5-BrdU mAb was made in Gratzner's laboratory by Abraham and Jeanne Stein. They developed a mAb against 7-methyl guanine with the imidazole ring cleaved under alkaline conditions (35,36). The objective of these studies, as yet unrealized, was to use this mAb as a reagent in determination and localization of the chemical methylation of in situ guanine as a probe of chromosome structure and DNA function.

During the early part of Leif's tenure at Coulter Corporation, he strongly recommended that Coulter exercise its rights under the agreement that created the Institute for Cell Analysis to commercialize Gratzner's anti-5-BrdU. He was working in the Coulter Applied Research group. Wallace Coulter and his new immunology guru, Dr. Top-Secret, were ending a discussion when Leif joined the conversation and brought up the subject of Gratzner's antibody. Leif argued that it would be a great reagent for Coulter's flow cytometers and sorters. Dr. Top-Secret

stated that he had a better technology, which of course, had to remain secret. Leif, although always outspoken, decided for once that discretion is the better part of valor and retreated to his office. This is the reason Leif never did any further work on anti-5-BrdU at Coulter Corporation.

Subsequently, the University of Miami had the vision to patent Gratzner's mAb against 5-BrdU and exercised its rights and licensed Becton Dickinson to manufacture and use the mAb. We are all gratified by the well-deserved commercial success of that product, its contribution to research in the cell cycle and cell kinetics, and its initial application for cancer chemotherapy assessment of the S phase. The ability to successfully apply for a patent takes not only some wisdom but also sufficient foresight with monetary backing. In this case, Becton Dickinson's commercial success with anti-5-BrdU showed the value of creative thinking and proper decision making. We are fortunate that Becton Dickinson and the University of Miami had the foresight to develop the product. Unfortunately, at the time, the internal problems of Coulter management had precluded profiting from the commercialization of the valuable intellectual property, the 5-BrdU mAb, which the company had so generously funded.

When the arrangement between Coulter and the Institute for Cell Analysis ended in 1981, Gratzner was again without funding. The one-third of the royalties paid to the University of Miami to support the Department of Medicine where Gratzner had been a member could not be used to support Gratzner because he now was a member of the Department of Microbiology and Immunology. Thus, Gratzner went first to Livermore, California and then to Texas. At Livermore he collaborated with Joe Gray's group which extended anti-5-BrdU research and showed the real power of this approach to study the cell cycle (37,38). They were able to simultaneously measure DNA content and 5-BrdU antibody staining. After about a year at Livermore, Gratzner went to Texas, where he collaborated with the cancer researchers and cell biologists at M.D. Anderson and with Bruce Cameron (a scientist at the PCRI), and further elucidated the cell cycle with the 5-BrdU assays.

Because of a personality conflict, Castro had already been transferred to an auxiliary campus of the University of Miami and remained there until his untimely death.

Leif, after leaving Coulter, moved to San Diego and has returned to working with anti-5-BrdU. The Phoenix Flow Systems kits for S phase and apoptosis, which are based on technology developed in Darzynkiewicz' laboratory (24,39), have been modified to use a europium macrocycle labeled with anti-5-BrdU (40).

Zucker left the PCRI, became a staff scientist at the U.S. Environmental Protection Agency in Research Triangle Park, North Carolina, and is operating a core flow cytometry facility and core confocal microscopy facility. Cell cycle analysis is a key component of his research.

#### **SIGNIFICANCE AND CONCLUSIONS**

It was not a trivial exercise to undertake this anti-5-BrdU project. However, if successful, the knowledge of

S-phase content and timing would be useful for the clinician to devise better therapy for the cancer patient. The use of complicated mathematical programs to decipher the cell cycle would no longer be needed; and cells in S phase could be more accurately measured with less effort. The flow cytometric aspects were easily achievable because excitation of propidium iodide and the fluorescein isothiocyanate antibody could occur with single argon ion (488 nm) laser, and the data could be displayed as a bivariate distribution. Most flow cytometers that existed in the later 1970s and early 1980s were capable of acquiring two-parameter fluorescence distributions. The scientists in Miami initially at the PCRI and then at the Institute for Cell Analysis worked on this project. They eventually delivered a technology that was capable of being transformed by industry into a commercial product that changed the way the cell cycle was studied.

The use of anti-5-BrdU is a procedure that has facilitated cell cycle studies and provided a model for the use of immunofluorescent reagents for the study of antigens in cell nuclei. The ultimate driving force behind these studies was to provide a better technique to help treat cancer. Scientists dedicated to using the new and exciting field of flow cytometry helped provide this valuable tool to allow other scientists to assess the cell cycle in various experiments using endpoints of microscopy and flow cytometry.

This useful work was accomplished by a group of scientists, each with complementary talents. There are valuable lessons to be learned from this anti-5-BrdU story. People do science often despite the institutional policies and quality of their institutional setting. The PCRI did not have sufficient funds to support this research or the necessary equipment or an administration with the vision to understand the significance of this work. In fact, the director refused to file a patent on this technology. It has to be admitted that our cooperation was due in part to the necessity of maintaining our funding, in part due to our desire to produce a useful technology for the medical and scientific community and perhaps, most importantly, to the ego trip associated with creativity. Credit also should be given to the national funding agencies, NIH and NCI, which had the vision to see the utility of the science. This work also would not have been completed without the generosity of Wallace Coulter.

A new technique to quantify cells in the S phase was created because a scientist had to solve a problem with his instrumentation. Conventional autoradiography required air drying from water, which was incompatible with maximizing the morphologic information used by the pathologist in making a diagnosis. We hope that this bit of history has demonstrated that it is not necessary for everyone to create all the hypotheses and new ideas. In a scientific research group, it also is necessary for some individuals to take a good idea and work on it; even though someone else initially proposed it. We hope we have succeeded in illustrating that, by interacting as a team, we were all able to bring about something useful, the development of this 5-BrdU assay.

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