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FIFTH INTERNATIONAL CONFERENCE ON IMMUNOFLUORESCENCE AND RELATED STAINING TECHNIQUES

Editors
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Morris Schaeffer

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FIFTH INTERNATIONAL CONFERENCE ON IMMUNOFLUORESCENCE AND RELATED STAINING TECHNIQUES*

Editors and Conference Chairmen

Willy Hijmans and Morris Schaeffer

Organizing Committee

T. E. W. Feltkamp, J. F. P. Hers, W. Hijmans, P. J. Hoedemaker, J. S. Ploem, and C. E. D. Taylor

The International Union of Immunological Societies Subcommittee on Immunofluorescence

A. Fagraeus, I. Batty, E. H. Beutner, W. B. Cherry, W. Hijmans, and E. J. Holborow

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* This series of papers is the result of a conference entitled Fifth International Conference on Immunofluorescence and Related Staining Techniques, sponsored by The New York Academy of Sciences and The Boerhaave Committee for Postgraduate Medical Education, assisted by The World Health Organization and The International Union of Immunological Societies, and held on July 17, 18, and 19, 1974 at The University of Leiden, Leiden, The Netherlands.

Part II. Antisera and Conjugates

Part III. Standards

Part IV. Model Systems

Part V. Quantitative Immunofluorescence

Part VI. Enzyme Methods

Part VII. Vital Staining Techniques in Cell Biology, Immunohematology, and Transplantation

Part VIII. Parasitology

Part IX. Bacteriology

Part X. Virology

Part XI. Venereal Diseases

Part XII. Hormones and Enzymes

Part XIII. Allergy

Part XIV. Autoimmunity

Part XV. Tumors

Part XVI. Immunopathology

GREETINGS FROM THE ACADEMY

Morris Schaeffer

Bureau of Biologics
Food and Drug Administration
Bethesda, Maryland 20014

On behalf of The New York Academy of Sciences, I bid a warm welcome to all gathered here to participate in the Fifth International Conference on Immunofluorescence. The Academy is proud once again to serve as a cosponsor to this illustrious event.

The fourth conference, held in Stockholm, May 13–15, 1970, was the first of these conferences to involve The Academy. Indeed, it was the first meeting of any kind ever sponsored by The Academy outside the United States.

In 1969, when Ernst Beutner first came to The Academy with the "unheard of" proposal for the Stockholm conference, I was the only microbiologist serving on the conference organizing committee. Impressed with the promise of such a meeting, I was able to convey my enthusiasm to my colleagues and we persuaded the Academy to "go international."

The huge success of that meeting, guided as it was by the capable and affable Astrid Fagraeus, is appropriately documented in Prof. Nairn's Concluding Remarks as "the greatest and most fruitful immunofluorescent congress of all time." These words and the remainder of the proceedings, so ably edited by Ernst Beutner, are indelibly recorded in the Annals of the New York Academy of Sciences (Vol. 177, June 1971).

Thus, it is not surprising that the proposal for the fifth conference from the Netherlands was received with such enthusiasm at The Academy. I am both pleased and honored to have been asked to assist in its development and I feel confident that this conference, following the established traditions, will equal, if not excel, those of the past. My contributions as cochairman are modest and perfunctory as compared to the imposing work of the local organizing committee.

Particular credit is due to Willy Hijmans, the "real" chairman of this Conference, for assuming the largest role in the preparation of the program and for the outstanding array of speakers about to be presented to you. We are indebted also to our hosts for making available these superb meeting facilities and for their generous provision of hospitality and creature comforts for the participants, during their sojourn in Leiden. Finally, we must not fail to mention our gratitude to Dr. Feltkamp and the Boerhaave Committee for their deligent and successful efforts to raise the funds that made this meeting possible.

INTRODUCTORY REMARKS

W. Hijmans

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It is a pleasure to welcome everyone on behalf of the organizing committee and also on behalf of the Boerhaave Committee for Postgraduate Medical Education of the Medical Faculty of the University of Leiden. We are grateful to The New York Academy of Sciences for their generous cooperation, which enables us to have this meeting in Leiden, and, on the personal level, to Dr. Morris Schaeffer, who acted as their representative. This meeting has received assistance by the World Health Organization, and this explains why it is the fifth conference of its kind. The first was a one-day meeting, convened in London in 1966 by the Medical Research Council at the instigation of the World Health Organization. Participation was limited to 25, and a mineographed report was made available. The main subject was the preparation of standardized antisera. The second meeting was held in 1967 in Florence, with the National Research Council of Italy as the major sponsor. Participation was international. Special topics were discussions on new light sources and the system of epi-illumination. The third meeting was held in London in 1968, under the auspices of the permanent Section for Microbiological Standardization of the International Association of Microbiological Societies. The pharmaceutical industry was represented for the first time. Proceedings of the second and third meetings have been published (1970. *Standardization in Immunofluorescence.* E. J. Holborow, Ed. Blackwell Scientific Publications. Oxford and Edinburgh.) The fourth meeting, held in Stockholm in 1970, received support from the Swedish Society for Microbiology and The New York Academy of Sciences. The applications of fluorescence in the various disciplines were discussed in great detail. The latest advances in technology, especially new filters, were also demonstrated. The proceedings have been published by The Academy (Ann. N.Y. Acad. Sci. Vol. 177. *Defined Immunofluorescent Staining*, E. H. Beutner, Ed.).

The present meeting will be recorded as the one with the greatest attendance so far. We were confronted with a choice between two evils: to invite a selected group, with the inherent danger of inbreeding, or to arrange an open meeting, with consequent logistic problems and difficulties in communication. We chose the latter, and whether this has been a wise decision remains to be seen. It means that we definitely need your cooperation in many respects to make this Conference run according to an already heavy schedule. It is also clear that we cannot proceed at the same tempo: a 12-fold increase in the number of participants in a period of eight years presents a curve that, on extrapolation, is a nightmare to any organizing body. We propose to submit this problem to the Subcommittee on Immunofluorescence of the International Union of Immunological Societies. Another characteristic of this meeting is the comprehensive exhibition by the manufacturers of microscopic equipment for immunofluorescence. As already mentioned, the World Health Organization has never failed to support these activities, and I would like to extend special thanks to their representatives here. The second international organization that lent support to this meeting is the International Union of Immunological Societies through its Subcommittee on Immunofluorescence. I believe that this

is its first such endeavor, and I hope that the meeting will live up to the high standards that the International Union of Immunological Societies has raised in the brief period since its initiation.

We are, furthermore, indebted to many sponsors, known and unknown. The known ones are:

World Health Organization; International Union of Immunological Societies; The Boerhaave Committee for Postgraduate Medical Education; Ministry of Education and Science, Organization for Health Research TNO; Central Laboratory of The Netherlands Red Cross Blood Transfusion Service; Netherlands Society for Immunology; Netherlands Asthma Foundation; Netherlands Heart Foundation; Netherlands League against Rheumatism.

Bio/Physics, Inc., Mahopac, N.Y.; Carl Zeiss, Oberkochen, Federal Republic of Germany; Ernst Leitz GmbH, Wetzlar, Federal Republic of Germany; Gist-Brocades NV, Delft, The Netherlands; Hoechst Pharma, Amsterdam, The Netherlands; Janssen Pharmaceutica NV, Beerse, Belgium; Merck, Sharp and Dohme Nederland BV, Haarlem, The Netherlands; Metals Research, Ltd., Royston, England; Miles-Yeda, Ltd., Rehovot, Israel; Nordic Immunological Laboratories, Tilburg, The Netherlands; Philips-Duphar BV, Weesp, The Netherlands; Philips Nederland BV, Eindhoven, The Netherlands; Reichert Optische Werke, Vienna, Austria; Schering Nederland BV, Weesp, The Netherlands; Vickers Instruments, York, England; Wellcome Nederland BV, Amsterdam, The Netherlands.

The unknown sponsors are the universities, institutes, and agencies that supported this meeting by granting travel awards to participants. They deserve special mention because these funds were made available to individual investigators, and funding in this case is an expression of confidence in the individual investigators and also in the organizing committee.

Immunofluorescence has come a long way since Reichert brought the fluorescence microscope on the market in 1911 and since the first publication by Coons and colleagues on this subject in 1941. It now has at its disposition a wide variety of tools, which range from simple microscopes to highly sophisticated machines. The same applies to the antisera, which have progressed from crude, poorly defined mixtures to highly purified antibody preparations. Of no lesser importance is the spin-off in the direction of immunoenzyme histo- and cytochemistry, which accordingly will be dealt with in detail during the present Conference.

It has been said before that the art of immunofluorescence has given way to the science of this subject. This development may entail a loss, but one should beware of nostalgia. If immunofluorescence had remained an art, it would not be the same today either.

The major point is that all of us will profit from this meeting, and we hope that we will have the art to enjoy the science of immunofluorescence.

GENERAL INTRODUCTION

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Leiden, The Netherlands

A series of international conferences have dealt with the standardization of reagents and technical procedures in immunofluorescence work. For a review of the general aims of these conferences, the reader is referred to the preface by Dr. Holborow in the published reports of the Florence conference[1] (1967) and the London conference (1968) and to the opening remarks by Dr. Fagraeus at the Stockholm conference[2] (1970).

Dr. Coons in his general introduction and Dr. Nairn in his concluding remarks at the Stockholm meeting reviewed the progress reported in defined immunofluorescent staining during the period between these conferences. In a recent paper, Faulk and Hijmans[3] have reviewed further progress in immunofluorescence (IF).

In this Leiden conference, we are looking forward to hear of more recent progress in immunohistochemistry, and more time than before is therefore reserved for the immunoenzyme (IE) techniques introduced by Avrameas, Nakane, Mason, and Sternberger and their colleagues.[4–7] Immunoenzyme techniques have enabled the extension of immunocytology to the level of the electron microscope. This has opened entirely new fields of research in immunohistochemistry.

The further development of standardized immunobiologic reagents is, however, essential for progress in the field of immunofluorescence and immunoenzyme methods.

This leads to the first point I should like to make. Whatever protein marker we use in immunohistochemistry, the physicochemical characteristics of the antigen–antibody interaction, the specificity, cross reactivity, and avidity of the antibody should always receive maximal attention, irrespective of which marker is used. Moreover, one should always pay attention to the presence or absence of competing antigens or antibodies of varying avidity in the preparation. In this context, it should be noted that the forces that bind antigen and antibody together are in essence not different from the so-called non-specific protein–protein interactions that occur between any two unrelated proteins.[8] Only the strength of the intermolecular forces will differ, depending on the closeness of fit.

In specimens where several antigens are present together with several antibodies, titers are the most important single criterion of the state of the antigen–antibody interaction. The complexity of such a system is often underestimated, and this may lead to an erroneous interpretation of the microscopic image. Most antisera, even those raised against antigens with a very simple structure, contain a variety of antibodies with a range of binding affinities.

In recent years, the evaluation of antigens and antibodies of different specificities in a single microscopic preparation has become more accessible through the simultaneous use of several fluorochromes or enzyme reaction products of various colors. Modern fluorescence microscopes enable a sensitive

detection of two fluorochromes, even in cases where the concentrations are widely different. Relatively weak red fluorescence from TRITC can be detected together with heavy staining with a green fluorescent FITC by the two-wavelengths excitation method. This technique has been applied successfully to the dynamic study of determinants on cellular membranes of living cells.[9-11]

Since immunoenzyme techniques may give staining reaction products of various colors, they can also be used for the simultaneous detection of more than one cellular constituent in fixed specimens.[12,13] Because the absorption spectra of such stains often overlap, the contrast obtained is generally less than with two different fluorochromes like FITC and TRITC, which have widely different absorption and fluorescence spectra.

Interpretation of the immunofluorescence of the immunoenzyme methods depends strongly on the specificity of antibodies used. This brings us to the second important point: the specificity testing of antibody preparations. During the last few years, many studies have been devoted to this problem. Through the immobilization of the antigen on an inert matrix, the fluorescent antibody technique can be used to assess immunological reactions.[14-16] At the Stockholm conference, Dr. Beutner stressed the need for such an approach to obtain a better analysis of antigen–antibody interaction in immunofluorescence.[17]

For the quantitative studies necessary in this context, there is a need for special carriers of fully defined but microscopic dimensions. Particles like Sepharose spheres, the diameter of which can be readily measured in the fluorescence microscope, are ideal for this purpose. In our laboratory, we have developed, in close cooperation with Dr. Hijmans and Dr. Haaijman of the Institute of Experimental Gerontology in Rijswijk, Dr. Knapp from Vienna, and Dr. Deelder of the Laboratory for Parasitology at Leiden, the Defined Antigen Substrate Spheres (DASS) system.[18,19] A similar approach was followed independently by Bergquist *et al.*[20] and Holubar *et al.*,[21] who used polymerized immunoglobulin particles. Capel[22] has also developed a sensitive system comparable to the DASS system. Streefkerk *et al.* have studied the possibilities of the Sepharose sphere system in enzyme immunohistochemistry.[23] The combined efforts of these groups have provided new tools for the quantitative study of antigen–antibody interaction in IF and IE.

The DASS system makes use of spherical agarose (Sepharose) beads to which a protein is covalently bound (FIGURE 1), according to the technique described by Axen *et al.*[24] When beads to which antigens are bound are incubated with the appropriate conjugates, a staining of the beads occurs, which can be measured for IF methods in a microfluorometer[19] and for IE methods in a scanning microdensitometer. Fortunately, Sepharose beads show very little autofluorescence with narrow-band blue (485 nm) and green (546 nm) excitation light, so that relatively weak fluorescence of a fluorochrome can also be detected. The sensitivity of the DASS system has been found to be on the order of a few nanograms of antibody per milliliter for certain antigen–antibody systems.[22,25,26]

The DASS system allows simultaneous testing of several antigens. This is possible by coupling a contrasting fluorochrome or an absorbing stain directly to the Sepharose beads prior to binding the antigen, which, in turn, will interact with its corresponding antibody in direct or indirect IF tests. The variation in prestaining serves as a marker of different antigens in simultaneous testing.

One fruitful application of the immunologic micromodel system is an improved definition of specificity of conjugates directed against the major

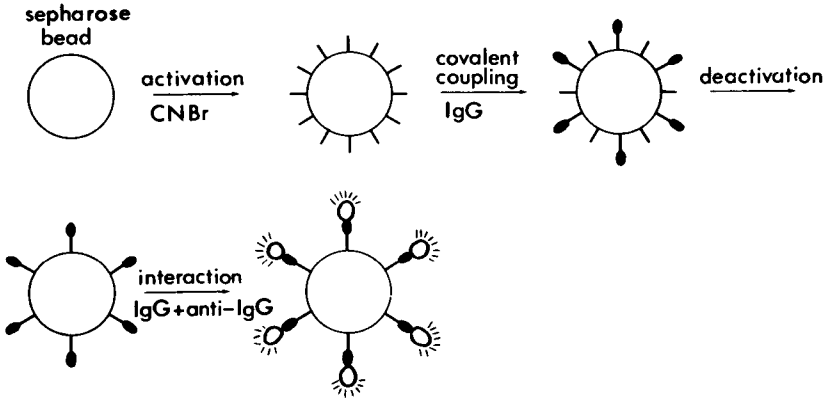

FIGURE 1. Scheme of covalent binding of IgG to a Sepharose bead and the subsequent interaction with anti-IgG.

human immunoglobulin classes. Bergquist and Kreisler[27] have conducted such a study, with spheres of polymerized albumen of microscopic size in which human immunoglobulins were incorporated. A similar study, with the DASS system, has been performed by Knapp *et al.*[28]

It may be concluded that micromodel systems can be used to characterize several properties of conjugates. They give information on specificity, nonspecific staining, and potency. An interesting possibility of Sepharose-matrices may be the possibility to introduce extra negative or positive charges to influence the extent of nonspecific staining.[26,32] They may serve therefore as a quality test system, especially when no performance test is available. Although the results with micromodel systems are promising, more data are needed to specify the upper limits of cross reactivity and nonspecific staining that are acceptable in the application of a given conjugate in a biologic system.

Studies conducted with model systems, although affording deeper insight into the relative magnitude of the different antigen–antibody interactions, do not replace performance testing of the conjugates with a variety of biologic substrates. The correlation between specificity testing of conjugates with the bone marrow system, as introduced by Hijmans' group,[29,30] and the results of a test on the same conjugates with the DASS system reported by Knapp *et al.*[28] will be of special interest.

An interesting possibility is the application of this micromodel system for practical diagnostic purposes, as has been demonstrated by Deelder *et al.*[19,31,32] for the immunodiagnosis of parasitic diseases in man. It must be stressed, however, that biologic substrates may possess antigenic determinants that as yet cannot be bound to a Sepharose bead. Capel[22,26] has indicated a promising new approach to solve some of these problems by binding hydrophilic antigens to Sepharose beads coupled to aliphatic amines.

The sensitivity of IF and IE is the next point that needs discussion. For a review of overall sensitivity of both IF and IE methods, the reader is referred to a paper by Avrameas.[33] He concludes that the overall sensitivity of both methods is about the same for a number of applications in light microscopy. In electron microscopic studies, the IE method requires a different definition of

sensitivity. This is related to the possibility to demonstrate extremely small amounts of antigens in ultrathin sections. The peroxidase–antiperoxidase complex method seems to be very sensitive and promising in this respect.[7]

In IF, the sensitivity depends on several factors that finally result in what may be called the "immunological contrast" in the specimen. One of these factors is the ratio between the desired specific staining (DSS) and the undesired specific staining (USS)[34] caused by cross-reacting antibodies. Another type of limitation of the sensitivity of the IF method is the nonspecific staining (NSS) of proteins in specimens with free fluorochrome. This can be largely avoided by controlled manufacturing of conjugates. Autofluorescence of biologic material can also reduce the sensitivity of the IF method. There are, however, cases where autofluorescence may be employed for orientation in the specimen.[35] With narrow-band blue (485 nm) and green (546 nm) excitation, the autofluorescence of biologic material is negligible in most specimens.[36]

A satisfactory "immunological contrast" comes out as a large image contrast and can therefore only be obtained if the desired specific staining is strong, the undesired specific staining weak, and the nonspecific staining and autofluorescence are negligible.

Quite different from the limitations of immunologic sensitivity in IF methods is the inability of a microscope to detect small concentrations of a fluroescent protein marker. Recent developments in fluorescence microscopy, such as the introduction of moderate power immersion objectives of high numerical aperture that optimally collect fluorescent light, e.g., a Leitz achromate oil immersion objective 40 x /NA 1.30 and a Zeiss apochromate 63 x /NA 1.40 in combination with wide-angle low-power eyepieces (4 x), afford the detection of much smaller quantities of fluorochrome than conventional systems. With modern microscope stands like the Leitz Orthoplan, the Reichert Univar, and the Zeiss Axiomat, large field-of-view numbers (more than 27) can be obtained that result in an acceptable viewing angle with low-power eyepieces. Conventional types of microscopes have only a field-of-view number of about 18, which results in a limited angle with low-power eyepieces, so that the microscopic image seems to be at the end of a tunnel. New optic developments, including improved filter systems, have made fluorescent images up to 1000% brighter than those obtained with conventional optic systems.

Enzyme methods have the advantage that only an ordinary light microscope is required and that strong staining reactions can often be obtained. The sensitivity of this method, however, is also limited by the "immunologic contrast" that is obtainable.

Miller[37] has reviewed factors that may limit "immunologic contrast" in IE methods. Nonspecific staining may be caused by endogenous activity of enzymes in tissues and nonspecific attachment of enzymes to biologic material that may be dependent on the type of fixation used. Further factors involved are the degree of diffusion of the enzyme and the substrate in the tissues and the preservation of antibodies and their diffusion. The sensitivity of detection in light and electron microscopy of the stained reaction product in IE is only restricted by certain fixatives and some counterstaining procedures.

In dynamic studies of living cells, such as lymphocytes and tissue culture cells, the IF method offers special advantages, because various microscopic methods often must be combined to preclassify the cells according to morphologic criteria. Phase-contrast or interference-contrast illumination with a substage condenser is used in combination with immunofluorescence microscopy using epi-illumination. IF methods are intensively used to investigate membrane

determinants of lymphocytes.[38–40] In this type of studies, two determinants on the cell membrane can be visualized with the aid of antibodies conjugated to fluorochromes of contrasting colors, such as FITC and TRITC.

In our studies with living cells, we use a newly developed type of reflection-contrast microscope[41] in combination with an inverted microscope equipped with a substage fluorescence epi-illuminator. We have obtained reflection-contrast micrographs from a human lymphoblastoid cell line (Moore 7002) that show numerous fine microextensions on the surface of the cell (FIGURE 2). The fine filaments visualized with this type of vital microscopy show a resemblance with the fine filaments seen in immunofluorescence micrographs of a preparation kindly provided by Dr. A. Fagraeus of cells of the same line treated with serum that contains SMA factor (FIGURE 3).

Since membrane fluorescence is often relatively weak, optimal filter combinations must be used.[42,43] Filter sets with very high transmittancies for excitation wavelengths that cause minimal amounts of autofluorescence are required.[36] Until now, one of the difficulties has been that secondary filters for the demonstration of weak FITC fluorescence on cellular membranes together with determinants binding TRITC conjugate were not available. A new combination of a conventional barrier filter (cut-off value at 515 nm) with a short-wave pass interference filter (SP 560, cut-off value at 560 nm) results in a secondary filter set[44] that stops all blue excitation light, transmits more than 70% of the green FITC emission peak, and stops the orange-red fluorescence of TRITC (TABLE 1, filter set 3). A second filter combination (TABLE 1, filter set 4) for the excitation of TRITC conjugates with green light does not excite any FITC but transmits more than 70% of the TRITC fluorescence.

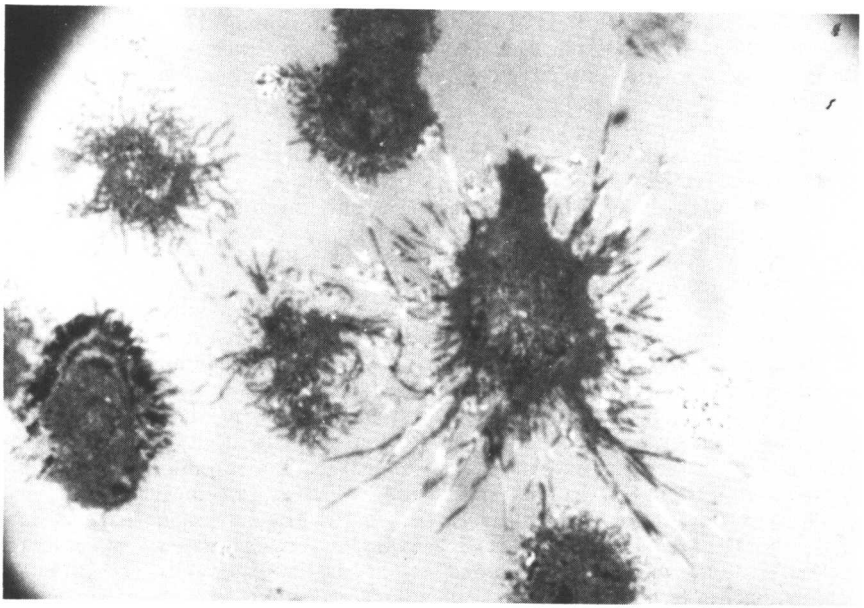

FIGURE 2. Reflection-contrast micrograph from a human lymphoblastoid cell line (Moore 7002). Notice numerous fine microextensions on the cell surface.