A SHORT HISTORY OF HISTOCHEMISTRY IN DENMARK

Published to celebrate the 40th Anniversary of
Danish Society for Cyto- and Histochemistry

Editorial Committee

Per Prætorius Clausen, Karina Norring Hjort,
Hans Oluf Lyon and Ole Nielsen

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THE BOARD'S PREFACE

DSCH is proud to celebrate its 40th anniversary. We are still an active scientific association. We find that there is great interest in our meetings, which, over the years, have changed. In the past our meetings were very research-oriented, whereas today we focus to a greater extent on the diagnostic relevance of using histochemical analyses.

Notwithstanding that histochemistry is a narrow scientific speciality in the larger scheme of things, Danish histochemists have exerted strong influence on the global development of their subject since the 19th century. Despite its diminutive size, DSCH is a vital association. Our members, scientific stakeholders and voluntary players are few but active and fervent by nature. Good things come in small packages – DSCH proves the point.

The Board wishes to express its thanks to **Per Prætorius Clausen**. Per is the brain behind this book and its very persistent editor, who saw the project to the door. Without Per, there would have been no book for all of us, on behalf of our specialism, to be proud of.

We are looking forward to passing on DSCH's high scientific standards to future generations and we are certain that we will have many glorious anniversaries to celebrate in the future.

The Board extends its thanks to everyone who has made an active contribution to our society through active membership or lectures or as active members of the Board. DSCH has survived and is today in an excellent state of health. This is your achievement.

On behalf of the Board

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EDITORIAL PREFACE
by Per Prætorius Clausen

On an occasion like this, it is tempting to paraphrase the statement regarding the Royal Danish Ballet by a former member of the Danish Parliament: *"Denmark is too small a language to have its own ballet"* and think that *" Denmark is too small to have its own histochemistry"*.

As it was relatively easy to repudiate the wisdom of the statement about the Royal Danish Ballet, we hope that this book will demonstrate that Denmark, despite its small size, has made and continues to make a remarkable contribution to the development of histochemistry – in Denmark and indeed also internationally.

The **Introduction** and scope in **Chapter 1** are followed by **Chapter 2**, which is an historical review of the development of histochemical staining techniques based on the biographies of seven Danish scientists, each of whom in his own unique way, contributed to these developments. **Adolph Hannover,** who unfortunately did not receive recognition for his colossally important scientific results until very late in life, cannot merely be described as "Denmark's first microscopist". He was a pioneer of histochemical methodology. **H.C. Gram**, **Frederik C.C. Hansen** and **Lárus Einarson** each lent their names to staining methods and modifications of them, to which their names continue to be associated. **Kaj Ulrik Linderstrøm-Lang, Heinz Holter** and **Niels Harboe** are names one would possibly not associate with histochemistry but the book reveals that each of these men exerted a strong influence on histochemical methods and their use.

Chapter 3 provides a comprehensive overview of modern histochemistry-related research with an introductory article about the pioneer of modern Danish histochemistry, **Helge Andersen,** and the cyto- and histochemistry laboratory he built. This portrait is followed up by an historical overview of histochemical methods used in neuroanatomy and brings examples of current research in this field.

Chapter 3 also includes personal accounts of the development of immunofluorescence staining in Denmark from its infancy in the 1960s and a brief account of the application of histochemistry in diagnostics, which now play a central and indispensable role in modern cancer diagnostics and therapy as described in the final section of Chapter 3.

Denmark is also at the forefront of international advances in method development, quality control and standardisation. Read **Chapter 4** for details.

It is a great pleasure that this book gives an opportunity to give a more comprehensive description of the substantial efforts devoted to teaching histochemistry in Denmark since the 1960s.

Chapter 5 describes developments in teaching and education of technicians as well as other biomedical assistants and scientists, and the tirelessness of the people who ensured the high technical standard of Denmark's laboratories.

Finally, **Chapter 6** explains an almost unnatural fact: that today, four decades after its foundation, Dansk Selskab for Cyto- og Histokemi (Danish Society for Cyto- and Histochemistry) is still alive and kicking. In an era, in which most professional exchange of ideas and knowledge takes place on the internet or at major international congresses, this incredibly tenacious little society has produced an impressive catalogue of activities throughout its lifetime. The list can be read as credits at the end of the chapter.

I wish to express sincere thanks to all the authors, who, at short notice, agreed to help make this book as broadbased a representation of our profession and subject as possible.

I also wish to thank the large number of people who have contributed in other ways to the publication of this book.

The editors have deliberately allowed the articles to alternate between articles that are personal and often amusing accounts and others that are relatively tedious, factual reports. The same has been true of Danish histochemistry down the years.

Finally my most heartful thanks to the board of the Danish Society for Cyto- and Histochemistry, who unreservedly and positively embraced the idea to publish this book to celebrate the society's 40th Anniversary.

On behalf of the Editorial Committee
Per Prætorius Clausen

INTRODUCTION
by Per Prætorius Clausen

Hans Christian Andersen published a fairytale entitled *Vanddråben (The Drop of Water)* in 1847. It is the story of Krible-Krable, a magician. One day, with his magnifying glass to his eye, he sits examining a drop of water taken from a puddle at the roadside. He observes thousands of tiny animals in it. *"At least I can give them a colour," he said, "so I can see them better." And then he let fall into the water a tiny drop of something that looked like red wine, but was really witches' blood, the very finest kind, and worth two pennies. Instantly the strange little creatures became red all over, and the drop of water now looked like a whole town full of naked wild men.* (1).

This was the first time that the staining of microscopic objects was described in a work of fiction (2). Hans Christian Andersen was undoubtedly inspired by a visit to a stately home, Hofmansgave, in northern Funen in 1830. In a letter to his friend, **Ludvig Læssøe**, on 15 July, he wrote: "*The last day I was in Hoffmansgave I had quite a treat, — I saw some infusoria. Just think, there was only One little drop of water on a piece of glass, and a whole world of creatures of which the largest looked like grasshoppers, the smallest as pins' heads... I saw infusoria in my own blood... the infinite opened before me, and I turned dizzy"* (3).

Figure 1. Part of an illustration in Ehrenberg's: *Die Infusionsthierchen als vollkommene Organismen.*

Posterity is, however, less sure of Andersen's source of inspiration for staining these animals. He may have been inspired by his attendance at a popular scientific lecture by the anatomist and physiologist, **D.F. Eschricht** at The Society of Natural History, which Eschricht himself had founded. Eschricht was in contact with the German naturalist, **C.G. Ehrenberg**, who in 1838 published a work on infusoria (4), which he stained by means of carmine (Figure 1). Ehrenberg had visited Copenhagen two years before (2). There's no doubt that the "witches' blood" that Krible-Krable used was in fact carmine.

A thought-provoking fact is that, in 1847, the year Hans Christian Andersen published his fairytale, one of the most important figures in the history of pathology (and a great deal more besides), **Rudolf Virchow** (1821-1902) (Figure 2), published in collaboration with his friend and colleague, **Benno Reinhardt,** the first issue of a journal, *Archiv für pathologische Anatomie und Physiologie und für klinische Medizin.* This journal is still published under the title *Virchows Archiv*.

In the first edition of the journal, Virchow made a frontal attack on the humoral pathological philosophy. Having originated in Ancient Greece, it was the predominant way of thinking in the mid 1800s, although in a modified form. Humoral pathology explained that the causes of disease were related to an imbalance of body fluids, the humors. In subsequent years, Virchow followed up his tirade and in 1858 he published a book: *Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebelehre* (5), which was hailed as the new canon of pathology (6). Virchow claimed that life processes were associated with the cells and the physiological and pathological processes that take place in them. He also believed that it was important to study these processes, which follow general physical and chemical laws. The pathologist's goal, he claimed, must be to create a physiological pathology, whereas the biologist's is to create physiological histology (7).

The next chapters will reveal that, to this day, it has been the histochemical methods in the broadest sense that have carried this goal forward, not only in histopathology, but also in cell biology generally.

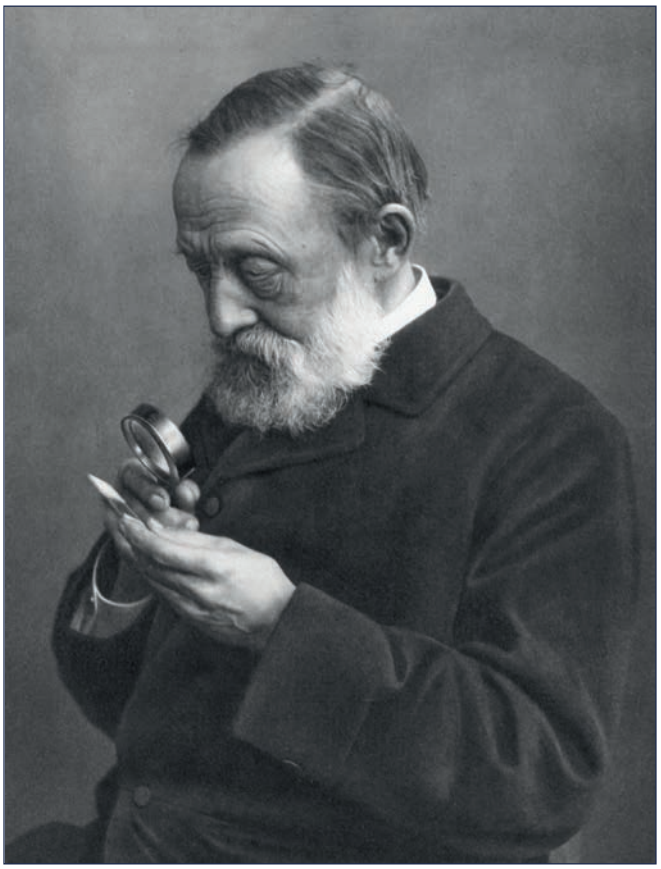

Figure 2. No, not Krible-Krable, although you would be forgiven for thinking it was. Rudolf Virchow, aged 70, examines a specimen on a slide under a magnifying glass.

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CHAPTER 1: WHAT IS HISTOCHEMISTRY?
by Per Prætorius Clausen

Histochemistry – from the Greek: ἱστός = tissue and Χῡμεία = the art of alloying metals – is a scientific discipline, which examines and describes the chemical composition of the cells and tissue of living organisms.

Microscopic histochemistry has the same purpose, although with the limiting factor that the presence of chemical constituent parts must be visible through a microscope.

By tradition, we distinguish between **histochemical** and **histological** methods. **Histochemical** methods explained the chemical composition and localisation of tissue components and insisted that the mechanism behind the reaction that made it visible was known. Meanwhile, **histological** methods sought primarily to visualise and distinguish between the constituent parts of tissue but made no requirements regarding chemical composition or a knowledge of the staining mechanism.

Over time, the distinction between these two methods was often revealed to be hazy and in the following, the term "histochemical methods" will be used to describe all the methods, which in some way have afforded information about the composition of cells and tissues and their structure and function (provided that the morphology is intact).

CHAPTER 2: THE DEVELOPMENT OF HISTOCHEMICAL STAINING METHODS

by Per Prætorius Clausen

2.1: THE DEVELOPMENT OF HISTOCHEMICAL STAINING METHODS UP TO THE MIDDLE OF THE 20TH CENTURY

Mankind has stained organic material since ancient times, when our forefathers dyed wool, fabric and items of clothing. The dyes most often used were natural dyes of animal or plant origin. A Danish book on dyeing from the 1700s (Figure 2.1.1) lists recipes for colouring with indigo from woad (Isatis tinctoria), cochineal extract from the bodies of female cochineal beetles, which contain a dye called carmine, and staining with chips from the logwood tree ((Hæmatoxylon campechianum), which was originally indigenous to Mexico and Central America, and contains haematoxylin.

In 1660, **Robert Hooke** (1635-1703) was the first to describe the structure of plants, which, seen under the microscope, appeared to be built of small structures, which he called *cells*.

The first to use staining in connection with his studies under the microscope was the Dutch textiles merchant and amateur lens grinder, **Antony van Leeuwenhoek** (1632-1723), who built the most powerful microscope of his time. In 1696, among many other objects, he studied muscle fibres from a duck's heart, which he stained with


En Dansk
Farve-Bog,
til
Almindelig Nytte,
eller
Kort Underretning
om
at farve Ulden, Silke og Linned,
tilligemed
Opskrift paa nogle Slags Blæk,
saa og
om at aftage alle Slags Plætter
paa Tøi.
Kiøbenhavn, 1768.
Bekostet af J. F. Heineck Mumme og Faber
i Bogladen No. 5. paa Børsen.

Fig. 2.1.1. At left:Title page of "En Dansk Farve-Bog (A Danish Dye Book)" from 1768; and at right: Wool dyed with indigo, cochineal and haematoxylin. (Wormianum, Per P. Clausen)

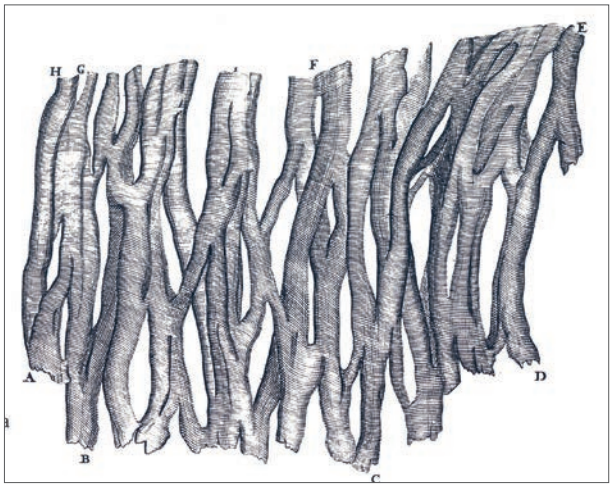
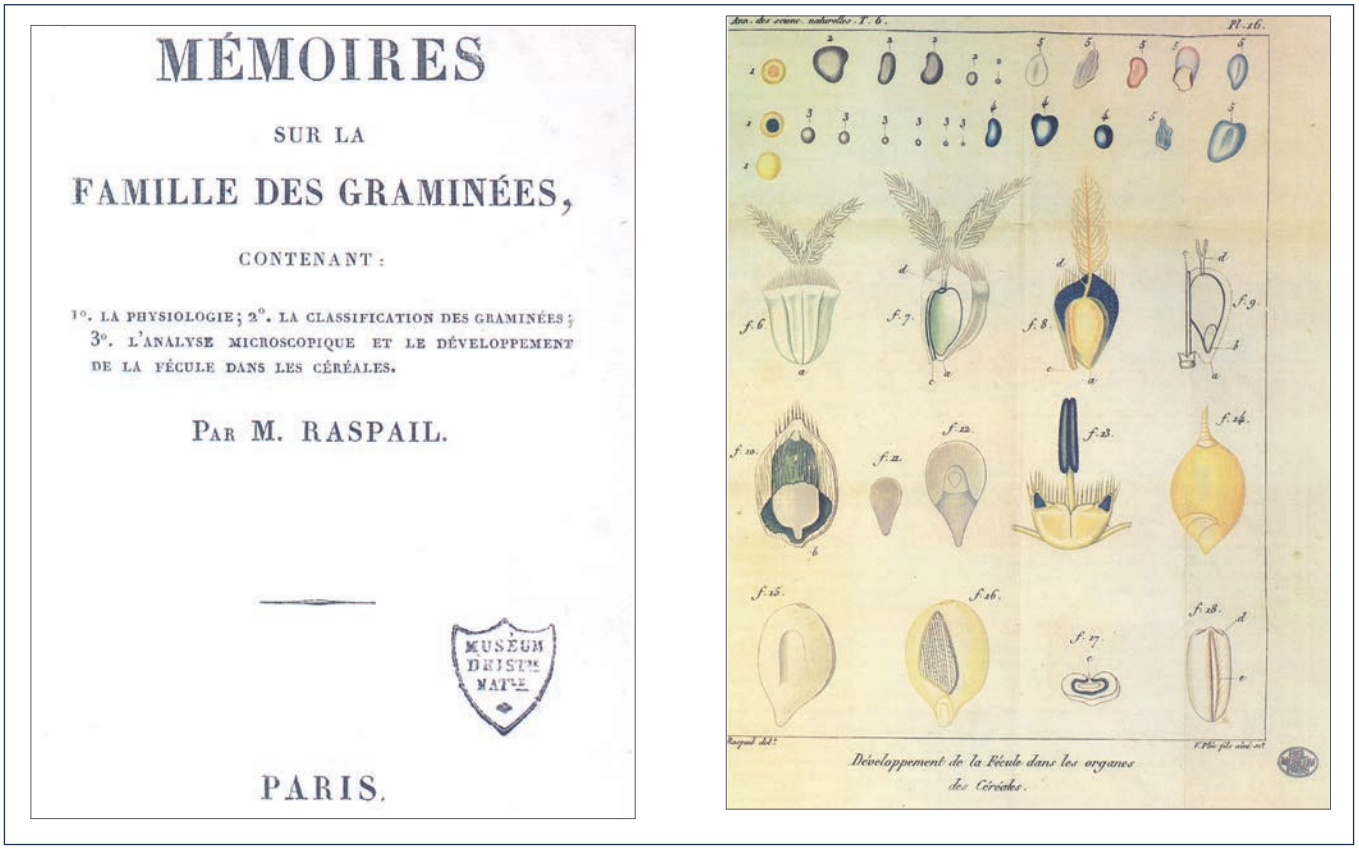


Fig. 2.1.2. Leeuwenhoek's drawing of the muscular fibres in a duck's heart.

saffron using crocus flowers soaked in wine (Figure 2.1.2). The botanist, **John Hill** (1714-1775) used an alcohol-based cochineal tincture to stain plant stems, which he then dissected into thin sections and examined under the microscope.

Before the early 1800s, microscopes were relatively poor, due to spherical and chromatic aberrations in the lens systems, which made images very blurred. In the 1800s microscopes were constructed with achromatic lens systems, which significantly improved the clarity of the images.

Histochemistry as a science was also founded in the early 1800s. From 1825 (1), a Frenchman, **Francois-Vincent Raspail** (1794-1878), performed extraordinarily original and comprehensive work, the results of which he published in 1830 in a book entitled: *Essai de Chimie Microscopique Appliquée à la Physiologie* (2). In this book, Raspail described how he had used iodine to detect the presence of starch in grain (Figure 2.1.3), listed other methods to detect the presence of proteins and carbohydrates in tissue and recommended freezing tissue before cutting it "to avoid damaging the tissue with the knife". Raspail's work received little attention and was soon overshadowed by launch after launch of new staining methods.


MÉMOIRES

SUR LA

FAMILLE DES GRAMINÉES,

CONTENANT :

1°. LA PHYSIOLOGIE; 2°. LA CLASSIFICATION DES GRAMINÉES; 3°. L'ANALYSE MICROSCOPIQUE ET LE DÉVELOPPEMENT DE LA FÉCULE DANS LES CÉRÉALES.

PAR M. RASPAIL.

MUSÉUM D'HIST^E NAT^LE

PARIS.

Développement de la fécule dans les organes des Céréales.

Fig. 2.1.3. The birth of histochemistry. In 1825 F-V. Raspail proved how starch develops in germinating seed using iodine staining, which coloured the starch blue. The drawings are Raspail's. (1)

Tissue preparation

The production of better microscopes brought with it more stringent demands on the quality of tissue preparation. Animal material has to be fixed in some way to both prevent autolysis and harden material to make samples suitable for microscopy. At that time, specimens were sliced manually using a barber's razor, which was either scraped over the surface or used to cut sections of tissue. The microtome was not in general use until the mid-1800s. **Klebs** described paraffin embedding in 1869. Alcohol was known and used as a fixing agent for many years. In 1832 and 1840, respectively, Danish scientists, **Ludvig Levin Jacobson** and **Adolph Hannover** (Box 2.1), described the first efficient fixation method (3) using diluted chromic acid to harden and also preserve tissue structure. A number of fixing agents were subsequently published, the most important of which are listed in **Table I.**

Primary fixing agents

Ethanol	Known since ancient times as a preservative	Used in histology since about 1743
Acetic acid	Known since ancient times as wine vinegar	Used in histology since 1663 (Robert Boyle)
Chrome trioxide	Hannover	1840
Mercury chloride	Blanchard	1846
Potassium dichromate	Müller	1860
Osmium tetroxide	Schultze	1864
Picric acid	Ranvier	1875
Formaldehyde	Blum	1893
Glutaraldehyde	Sabatini	1963

Compound fixatives

Clarke	1851	Bouin	1897
Flemming	1882	Heidenhain SUSA	1916
Carnoy	1887	Karnovsky	1965
Zenker	1894	Stefanini (Zamboni)	1967

Table I. Primary fixative agents and compound fixatives, who first described them and when.

Adolph Hannover

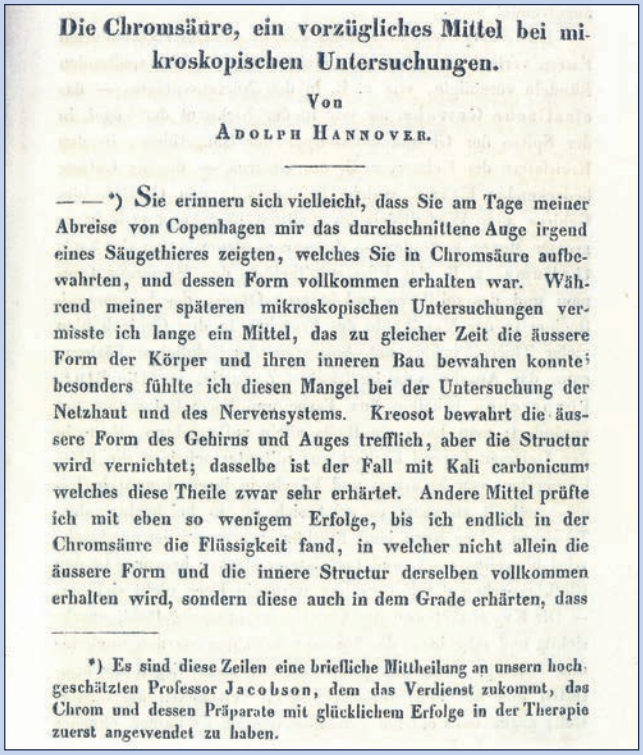
(1814-1894)

Figure 1. Adolph Hannover pictured in 1854. Lithography by Fortling after a painting by Monies. (From "Adolph Hannover og hans fædrene og mødrene slægt" (Adolph Hannover and his father's and mother's family trees), published by Martin Ad. Hannover, Copenhagen 1914)

Box 2.1

Adolph Hannover (Figure 1) is often described as "Denmark's first microscopist". This description is not quite correct but there is little doubt that he was Denmark's first histopathologist. He finished school in 1832 and began to study natural history. He met a fellow student, **Japetus Steenstrup** (1813-1897), who later became professor of zoology. The two men became friends for life. Hannover switched from natural history to medicine. Even in his student days, Hannover received accolades. He was awarded a gold medal from the university for a paper about his microscopic examination of the ganglia in the nervous system. He graduated in 1838 and in 1839 he received his Ph.D. for a comparative anatomic study of the outer ear. As early as 1832, in connection with a study of the physiological effect of chromic acid and its salts, his friend and much older colleague, **Ludvig Levin Jacobson** (1783-1843) found that this particular substance has a characteristic that is particularly important for anatomy and the natural sciences, i.e. *"a very weak solution of this salt can be used to preserve the objects one wishes to examine or form part of a collection"* (1). This is history's first description of adequate fixation (2).

Armed with this knowledge, in 1839, Hannover set off on a two-year study tour of Europe, which brought him to both Berlin and Paris. His visit to Berlin and his meeting with anatomist and physiologist **Johannes P. Müller** (1801-1858) were decisive. During this visit, Hannover passed on L.L. Jacobson's studies of the use of chromic acid as a fixative agent and published the results in Müller's journal in 1840 (Figure 2) (3).



Die Chromsäure, ein vorzügliches Mittel bei mikroskopischen Untersuchungen.

Von

Adolph Hannover.

— —*) Sie erinnern sich vielleicht, dass Sie am Tage meiner Abreise von Copenhagen mir das durchschnittene Auge irgend eines Säugethieres zeigten, welches Sie in Chromsäure aufbewahrten, und dessen Form vollkommen erhalten war. Während meiner späteren mikroskopischen Untersuchungen vermisste ich lange ein Mittel, das zu gleicher Zeit die äussere Form der Körper und ihren inneren Bau bewahren konnte; besonders fühlte ich diesen Mangel bei der Untersuchung der Netzhaut und des Nervensystems. Kreosot bewahrt die äussere Form des Gehirns und Auges trefflich, aber die Structur wird vernichtet; dasselbe ist der Fall mit Kali carbonicum, welches diese Theile zwar sehr erhärtet. Andere Mittel prüfte ich mit eben so wenigem Erfolge, bis ich endlich in der Chromsäure die Flüssigkeit fand, in welcher nicht allein die äussere Form und die innere Structur derselben vollkommen erhalten wird, sondern diese auch in dem Grade erhärten, dass

*) Es sind diese Zeilen eine briefliche Mittheilung an unsern hochgeschätzten Professor Jacobson, dem das Verdienst zukommt, das Chrom und dessen Präparate mit glücklichem Erfolge in der Therapie zuerst angewendet zu haben.

Figure 2. The title page of Hannover's description of chromic acid as a fixing agent. The paper is written as a letter to his mentor L.L. Jacobson. (1)

When he returned home, Hannover began his clinical training at Frederik's Hospital. During this period, he performed a series of microscopic examinations of the eye and the nervous system, which were published in *Mikroskopiske Undersøgelser af Nervesystemet (Microscopic Studies of the Nervous System)* in 1842. This paper is beautifully illustrated by Hannover himself (Figure 3). In 1847, he published a book about the microscope, *Om Mikroskopets Bygning og dets Brug (On the construction and use of the microscope)*, which was translated into English, German, French and Dutch. In another work, *Om Epithelioma, en særegen Svulst (On Epithelioma, an abnormal growth)* (1852), he was the first in Denmark to recommend taking a biopsy in connection with diagnostics: *"The microscope can with benefit be used before the tumour is removed from the body, as we can obtain small specimens for examination... Although we should not have to settle for very superficial parts of the tumor"*.

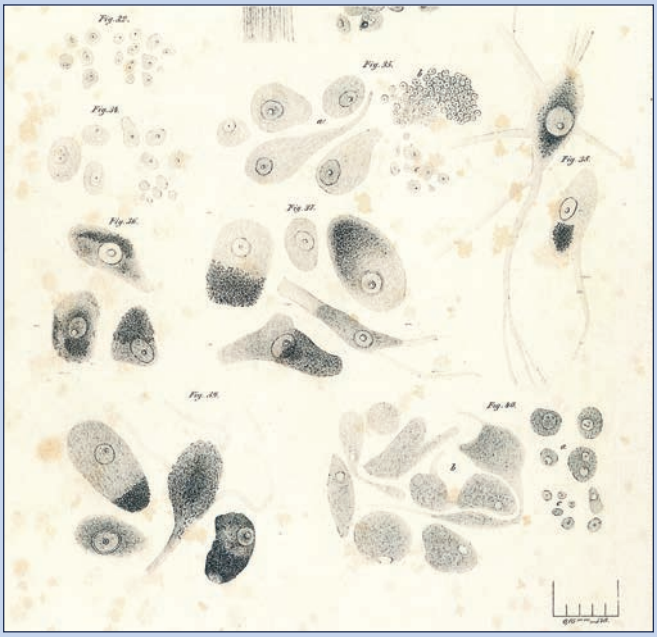


Figure 3. Excerpt from an illustration in Hannover's book "Mikroskopiske undersøgelser af nervesystemet" (Microscopic studies of the nervous system), 1842

From this time almost until his death, Hannover was a prolific author of scientific works covering a wide range of subjects including pathology, microbiology, medicine, medical statistics, invalidity, poverty and hygiene. Despite his scientific fervour, he did not succeed in obtaining a permanent position at The University of Copenhagen, neither in 1843, when he applied for a job as lecturer in pathological anatomy, nor in 1846, when he applied for a job as lecturer in descriptive and microscopic anatomy. He earned a living working as a general practitioner and as a medical office at Copenhagen hospitals and as a military and cholera physician.

He received several awards from abroad, and, at the international medical congress in Copenhagen in 1884, of all the Danish physicians, he was the one best known to foreign attendees. (During the congress, he was host to **Sir James Paget** (1814-1899), who, in addition to being a surgeon, was also one of England's leading pathological anatomists. Paget lent his name to several diseases of the mammae and bones).

An auditorium at the Panum Institute at the University of Copenhagen was named after Hannover in 1984.

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Staining with carmine and haematoxylin

As described above, carmine has long been recognised and used to stain preparations for microscopy. In the first half of the 19th century, carmine was almost synonymous with histological staining. **Alfonso Corti** (1822-1876), who is known for his studies of the inner ear and, from 1851, the organ that bears his name, used chromic acid to fixate and harden and a carmine solution to stain cell nuclei. In 1858, **Joseph von Gerlach** (1820-1896), accidentally developed the perfect recipe for carmine staining (4). He left a cerebellum, fixated with potassium dichromate, in an extremely dilute solution of carmine overnight and succeeded in producing the finest nucleic staining (Figure 2.1.4). He discovered that staining did not work on unfixed tissue, which indicated that the chrome fixative was required to mordant the stain. Carmine staining persisted into the 1880s as the histological staining method of preference. Later modifications of the method were used to stain mucus: Mucicarmine (Figure 2.1.5).

The textile dyeing industry had discovered at an early stage that if you wished to use haematoxylin as dye, the chips of logwood had to be be soaked and exposed for several days to light and air to achieve optimal results. In addition the fabric had to be pretreated with alum. **F. Böhmer** used the dyers' expertise when he described his alum-haematoxylin staining method in 1865 (5). However, it was not until 1891 that **Paul Mayer** (1848-1923) discovered that the active ingredient, i.e. the chemical compound that dyes, is an oxidation product of haematoxylin called haematein, and that haematin can be obtained by adding e.g. iodate (6). Around the turn of the century, **F.C.C. Hansen** (Box 2.2) tested a large number of haematoxylin protocols and published his own iron haematoxylin staining method. By the end of the 1800s, haematoxylin staining methods were the preferred methods for staining nuclei.

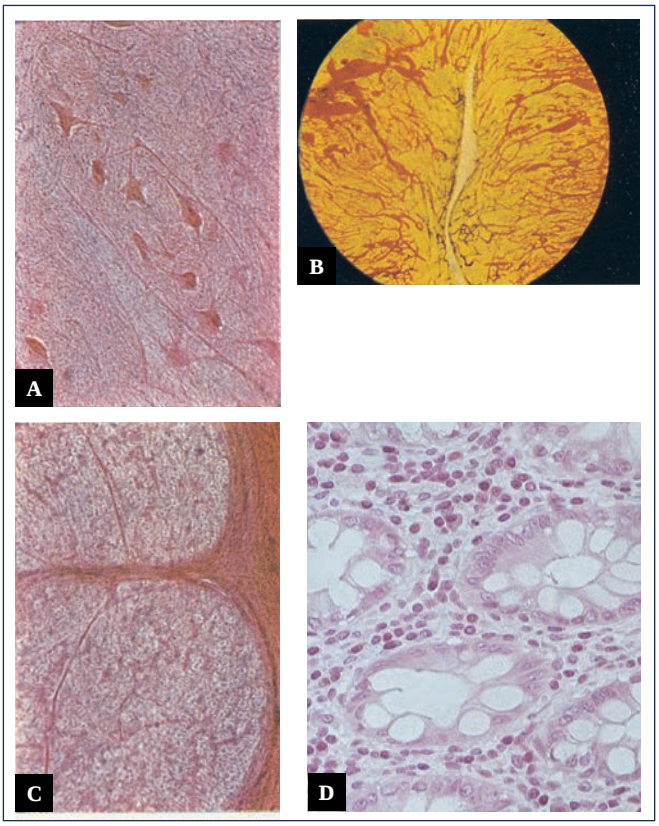


Figure 2.1.4: Carmine staining **A+C**: Images of a section of medulla spinalis, cut by hand and stained about 1860. The slide was found at the Department of Anatomy at the Danish Academy of Surgeons (now Medicinsk Museion) in 1942 and is attributed to Goll in Zürich. **B**: Section of uterine mucosa, in which the blood vessel is injected with carmine gelatin. (Reproduced from a plate in Harald Okkels: Farveteknikken i den mikroskopiske anatomi (Staining techniques in microscopic anatomy), Copenhagen 1947). **D**: Section of colonic mucosa stained in 2016. (Janne Jensen)

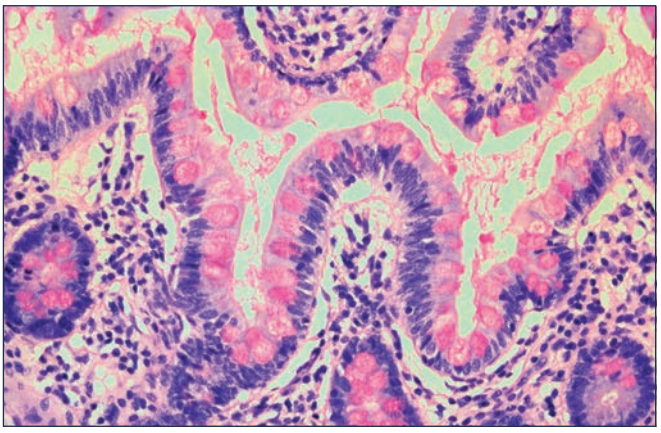


Figure 2.1.5 Mucicarmine stain of small intestine mucosa (Lene Lütken Sørensen, Jess Pilgaard)

Frederik Carl Christian Hansen (1870-1934)

Figure 1. Frederik C.C. Hansen. (Hospitalstidende, 6 February 1934)

Box 2.2

Frederik Carl Christian Hansen (Figure 1) became a doctor in 1894. As undergraduate and postgraduate, he was interested in microscopic anatomy. As a postgraduate student, he studied under **Rudolph Sofus Bergh**, a zoologist, who was associate professor of histology and embryology and the first to introduce microscopy into zoology education. In 1897, Fr. C. C. Hansen was appointed prosector of anatomy at the university, where he continued his studies of microscopy and histological technique.

In 1900, he wrote a thesis: *Undersøgelser over Bindevævsgruppen, 1. del, Den hyaline Bruskgrundsubstans (Studies of the Connective Tissue Group, Part 1, Hyaline, an essential ingredient in cartilage)*. He became professor of anatomy in 1901. At this time, his main field of interest was the technical issues related to histological staining, especially haematoxylin staining. In 1905, he published a review (1) in which he describes his version of iron haematoxylin staining, which has since (particularly in Denmark) been used to stain nuclei in conjunction with Van Gieson's picrofuchsin staining to distinguish between muscular and connective tissue. In Denmark, this method is known as Van Gieson-Hansen's stain (Figure 2).

As professor, F.C.C. Hansen incorporated histology much more in his teaching than previously had been the case – without a corresponding reduction in the macroscopic anatomy curriculum. He was therefore not too popular with his students. From 1908, he switched to a new main scientific field of interest and published a number of anthropological works.

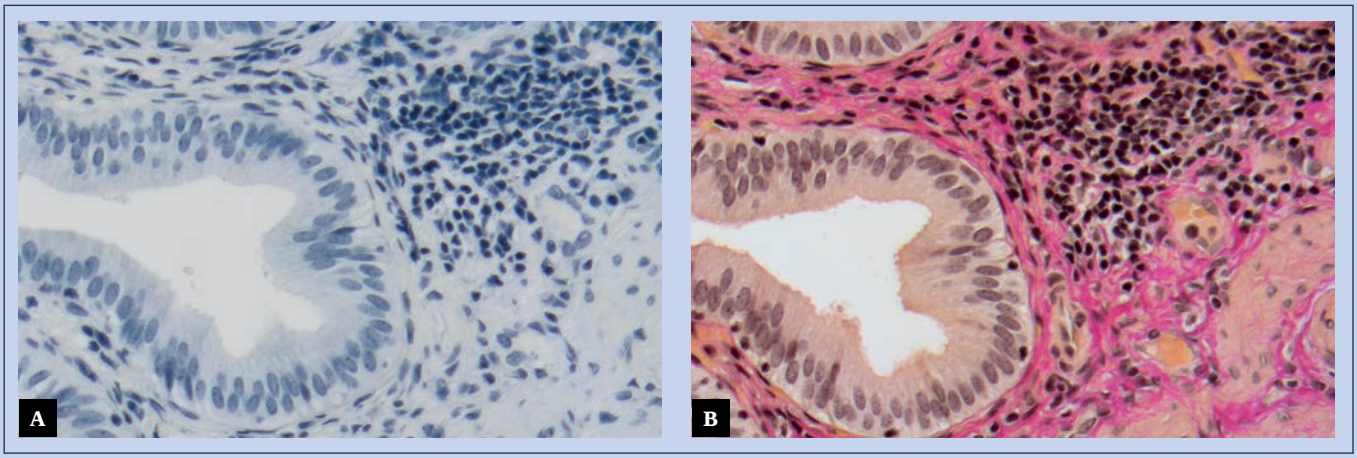


Figure 2. Iron haematoxylin staining. A: Stained only with iron haematoxylin ad modum Hansen. B: Same staining used in combination with Van Gieson's picrofuchsin staining (1889), which dyes collagen red and muscular tissue yellow. (Ole Nielsen)

Reference list

1. Hansen FCC. Über Eisenhämatein, Chromalaunhämateïn, Tonerdealaunhämateïn, Hämateïnlösungen und einige Cochenillefarblösungen. Zeitschrift für Wissenschaftliches Mikroskopie und mikroskopische Technik 1905;22:45-90.

Aniline dyes and everything that followed...

At Easter (March) 1856, 18-year-old **William Henry Perkin** (1838-1907) was trying to synthesise quinine, a substance which the British desperately needed to fight malaria in the colonies. He succeeded in producing aniline as an extract of nitrated benzene. He failed to make quinine on this occasion but successfully produced a purple dye called mauvein. The textiles dyeing industry was jubilant!

It was the first synthetic dye ever produced and it gave the chemicals industry a kick-start too. The discovery was soon also used in histological staining. Mauve was used first in 1862 and then there was a major increase in the production of synthetic dyes, not only aniline derivates, but also a large number of other synthetic dyes. **Table II** is a list of selected dyes, which are used in histology.

In 1902, **Gustav Mann** described the period: *" The method of staining,…grew and grew, until to be an histologist became practically synonymous with being a dyer, with this difference, that the professional dyer knew what he was about, while the histologist with few exceptions did not know." (7).*

Perkin's mauve	1862	Beneke
Paris blue	1863	Waldeyer
Aniline blue	1863	Waldeyer, Frey, Roberts
Basic fuchsin	1863	Roberts
Picric acid	1863	Roberts
Indigo carmine	1864	Chrzonszczewsky
Cyanine	1874	Ranvier
Iodine violet	1874	Zuppinger, Huguenin
Alizarin	1874	Lieberkühn
Methyl violet	1875	Cornil
Eosin	1875	Fischer
Safranin	1877	Ehrlich
Methyl green	1877	Calberla
Bismarck brown	1878	Weigert
Methyl blue	1879	Ehrlich
Nigrosin	1879	Ehrlich
Aurantia	1879	Ehrlich
Tropaeolin	1879	Ehrlich
Acid fuchsin	1879	Ehrlich
Orange G	1879	Ehrlich
Bordeaux	1879	Ehrlich
Methylene blue	1880	Ehrlich
Biebrich scarlet	1880	Schwarze
Iodine green	1881	Stirling, Richardson
Magdala red	1881	Flemming
Chrysoidine	1883	Griesbach
Malachite green	1884	Beneden and Julin
Erythrosin	1885	Gierke
Thionin	1885	Ehrlich
Light green	1886	Griesbach
Congo red	1886	Griesbach
Benzopurpurin	1886	Griesbach
Toluidine blue	1890	Hoyer
Neutral red	1893	Ehrlich
Sudan III	1896	Daddi
Janus green	1898	Ehrlich
Sudan IV	1901	Michaelis
Azocarmine	1905	Heidenhain
Nile blue	1908	Smith
Trypan blue	1909	Goldmann

Table II. Table showing selected dyestuffs, who first described their use in histology and when (3).

One of these exceptions was **Paul Ehrlich** (1854-1915). In 1877-1880, he performed a systematic investigation of the aniline dyes and described the principal difference between acid and alkaline dyes, chemically and histologically (8,9). He introduced the term "metachromasia", discovered that tubercle bacilli are acid-fast, and devised many different staining methods.

A significant number of the staining methods described in the second half of the 1800s are still in use. Figure 2.1.6 is a colourful overview of some of these, including Gram Staining (Box 2.3).

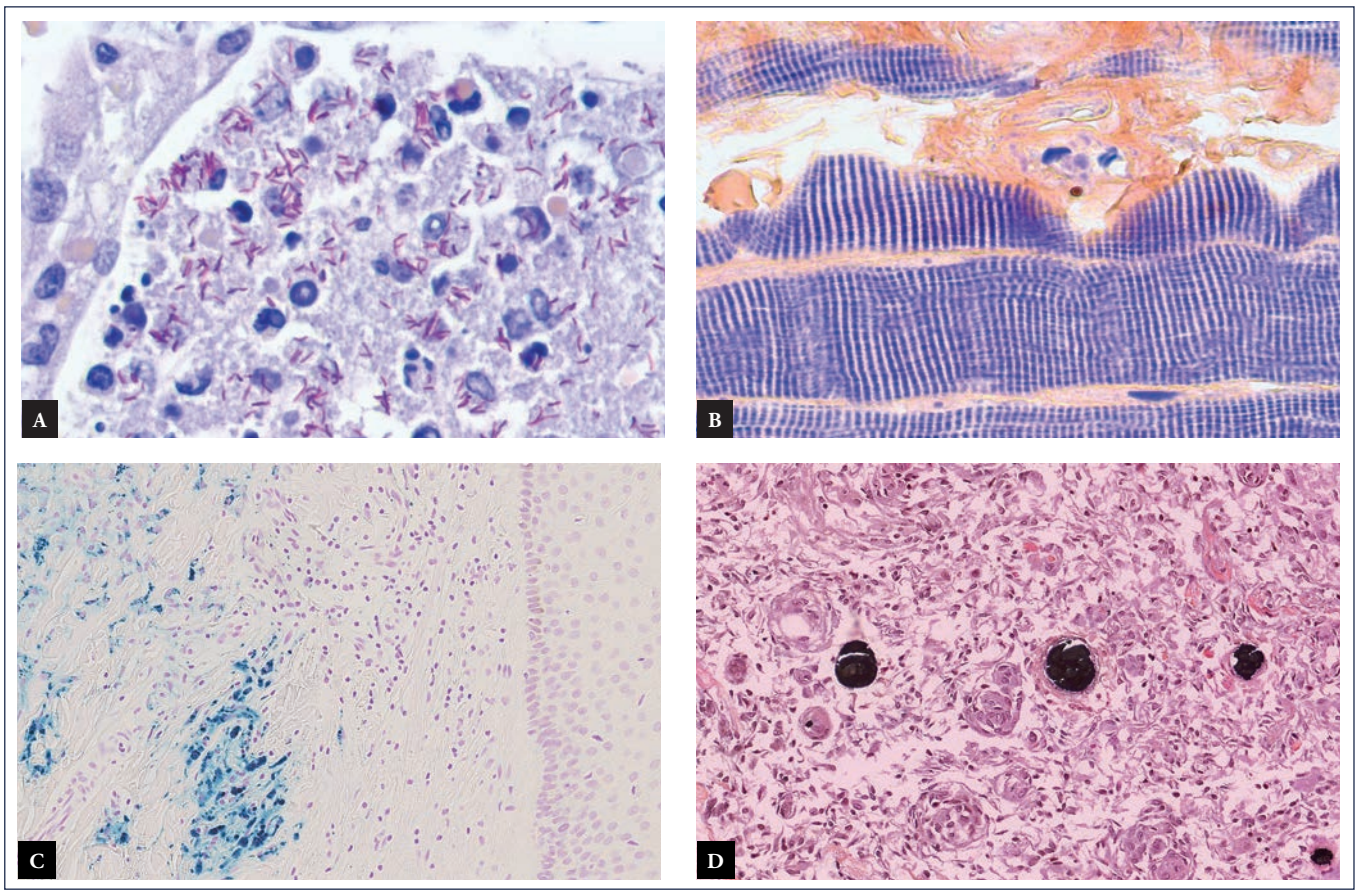


Figure 2.1.6 Plate showing selected staining methods, which were described in the second half of the 1800s and which are still used today. **A**: The Ziehl-Neelsen Stain (1882/1883) of acid-fast bacilli. **B**: Mallory's PTAH stain (1897), which demonstrates striations in skeletal muscle tissue. **C**: Perls' staining (1867) for iron pigment and **D**: The von Kossa staining (1901) for calcium. (Ole Nielsen)

Hans Christian Joachim Gram (1853-1938)

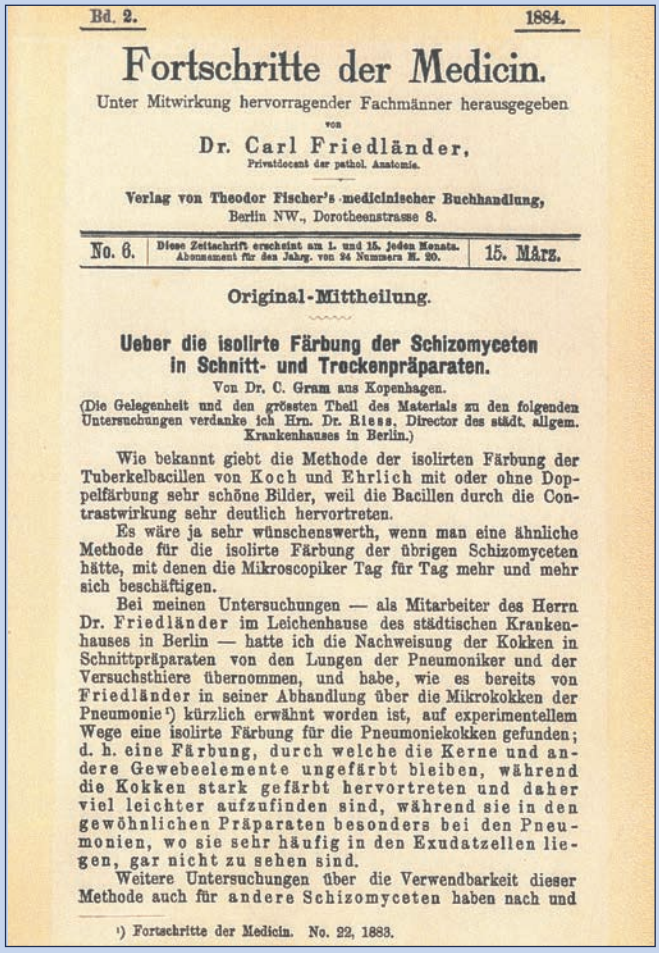
Figure 1. Christian Gram photographed in the 1880s when he was working on his staining method. (Medicinsk Museion)

Box 2.3

After graduating from school in 1871, **Hans Christian Joachim Gram** (Figure 1) began reading natural history at university and took the pre-medical examination at the same time. He attended **Japetus Steenstrup's** lectures. Steenstrup encouraged Gram to study medicine, which he did, but he continued to work as Steenstrup's assistant at the Zoological Study Collection, where he learned a great deal about microscopy. After he qualified as a doctor of medicine in 1878, he wrote a gold-medal thesis (1882) and a Ph.D thesis (1883).

Both works addressed determining the size of the red blood corpuscles under the microscope and Gram received praise for his precision. As a postgraduate, he attended a bacteriological course taught by the Danish pioneer of bacteriology, **Carl Julius Salomonsen**. On Salomonsen's recommendation, in 1883 Gram travelled to Berlin to study under **Carl Friedländer**, who was prosector at the Institute of Pathology at Städisches Allgemeines Krankenhaus. Friedländer hoped to find the cause of pneumonia and Gram was given the task of staining and examining tissue specimens from patients, who had died of pneumonia.

In connection with this work, Gram very soon succeeded in developing a staining method for detecting pneumococci, which posterity has named after him (1). He wrote that he discovered the method by chance. He was trying to make a double staining of a section of kidney tissue, in which the nuclei were to be dyed blue and the urine casts brown. : This is one of several examples in the history of science called *serendipity*: *Making an important discovery while searching for something else.* The discovery of penicillin is another example. Gram discovered a staining method, which he described as follows: *"nuclei and other tissue elements are not coloured while the cocci appear vibrantly dyed and therefore much easier to see, whereas in the customary preparations especially for pneumonia, they often lie in exudate cells and are invisible"*. The staining method was published in Friedländer's journal (Figure 2).



Bd. 2. 1884.

Fortschritte der Medicin.

Unter Mitwirkung hervorragender Fachmänner herausgegeben von

Dr. Carl Friedländer,
Privatdocent der pathol. Anatomie.

Verlag von Theodor Fischer's medicinischer Buchhandlung,
Berlin NW., Dorotheenstrasse 8.

No. 6. | Diese Zeitschrift erscheint am 1. und 15. jeden Monats. Abonnement für den Jahrg. von 24 Nummern M. 20. | 15. März.

Original-Mittheilung.

Ueber die isolirte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten.

Von Dr. C. Gram aus Kopenhagen.

(Die Gelegenheit und den grössten Theil des Materials zu den folgenden Untersuchungen verdanke ich Hrn. Dr. Riess, Director des städt. allgem. Krankenhauses in Berlin.)

Wie bekannt giebt die Methode der isolirten Färbung der Tuberkelbacillen von Koch und Ehrlich mit oder ohne Doppelfärbung sehr schöne Bilder, weil die Bacillen durch die Contrastwirkung sehr deutlich hervortreten.

Es wäre ja sehr wünschenswerth, wenn man eine ähnliche Methode für die isolirte Färbung der übrigen Schizomyceten hätte, mit denen die Mikroscopiker Tag für Tag mehr und mehr sich beschäftigen.

Bei meinen Untersuchungen — als Mitarbeiter des Herrn Dr. Friedländer im Leichenhause des städtischen Krankenhauses in Berlin — hatte ich die Nachweisung der Kokken in Schnittpräparaten von den Lungen der Pneumoniker und der Versuchsthiere übernommen, und habe, wie es bereits von Friedländer in seiner Abhandlung über die Mikrokokken der Pneumonie [1]) kürzlich erwähnt worden ist, auf experimentellem Wege eine isolirte Färbung für die Pneumoniekokken gefunden; d. h. eine Färbung, durch welche die Kerne und andere Gewebeelemente ungefärbt bleiben, während die Kokken stark gefärbt hervortreten und daher viel leichter aufzufinden sind, während sie in den gewöhnlichen Präparaten besonders bei den Pneumonien, wo sie sehr häufig in den Exudatzellen liegen, gar nicht zu sehen sind.

Weitere Untersuchungen über die Verwendbarkeit dieser Methode auch für andere Schizomyceten haben nach und

[1]) Fortschritte der Medicin. No. 22, 1883.

Figure 2. Title page of Christian Gram's description of his staining method for detecting bacteria (1).

Gram tested his method on many different types of inflammations and discovered that while, in some cases, bacteria were stained, in others they were not. **Weigert** later supplemented Gram's original protocol by adding safranin, the effect of which was to stain red the bacteria that remained uncoloured in Gram's method. This method, which differentiates between two, unrelated groups of bacteria, Gram-positive and Gram-negative, was later – and continues to be – extremely important for diagnostics and differential diagnostics (Figure 3).

When Gram left Friedländer and Berlin after only four months, he also quit bacteriology. In May 1884, he stayed with pharmacologist **Oswald Schmiedeberg** in Strasbourg and subsequently devoted his life to pharmacology. On his return to Denmark in 1891, he was appointed professor of pharmacology and senior physician in the medical department at Frederik's Hospital. In 1900, he was appointed professor of medicine, a position he held until he retired in 1924.

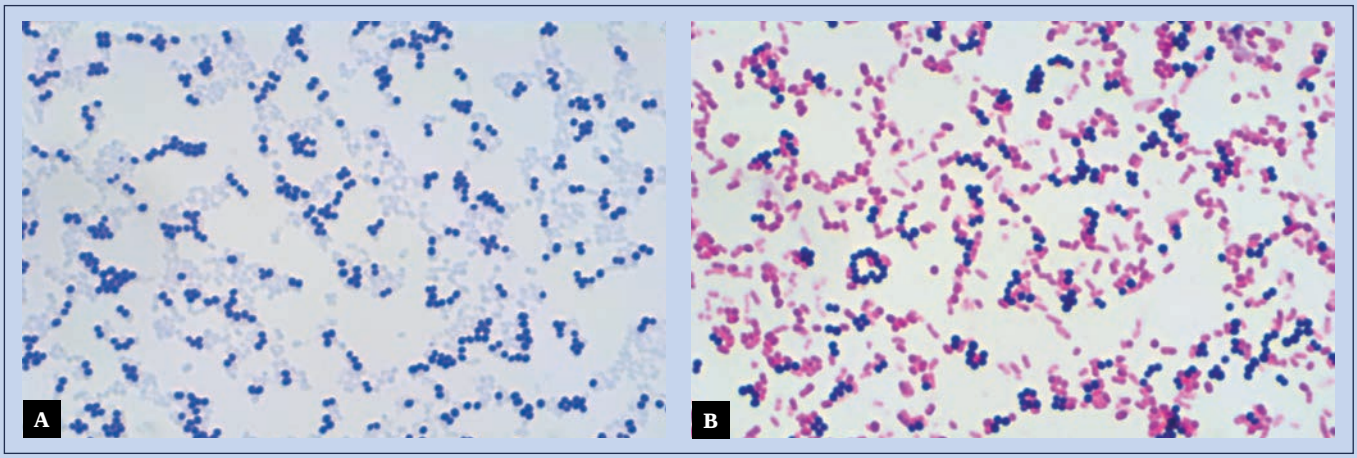


Figure 3. A. The Gram Stain, as Gram himself described it. Only Gram-positive bacteria are stained blue. **B**: Gram stain counter-stained ad modum Weigert. Gram-positive bacteria are blue, Gram-negative red. (Sanne Malig, Louise Pedersen, Ole Nielsen)

Reference list

1. Gram HC. Ueber die isolirte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. Forschritte der Medicin 1884;2:185-9.

By the end of the 19th century, science could look back on a period, in which cell theory had been founded. Scientists had established that all plants (**Schleiden**, 1838) and animals (**Schwann**, 1839) were built of cells. **Virchow** coined his aphorism: *"Omnis cellula e cellula"* or "all cells come from cells" to describe how tissue is formed. He published this in a paper *Cellularpathologie* in 1855 (10). **Raspail** had already described this in 1825 (1) but had been forgotten for 40 years.

Towards the end of the century, most cell organelles had been described: Endoplasmic reticulum (1897) and mitochondria (1898). **Camillo Golgi** described his eponymous apparatus in the same year. The lysosome was first described and named by **Christian de Duve** in 1955. He received the Nobel Prize for his work in 1974.

In 1879, **Walther Flemming** gave the name "chromatin" to the substance in cell nuclei, which could be stained using alkaline dyes. He also described and gave the name to "mitosis" and reworded Virchow's aphorism: *"omnis nucleus e nucleo"*. **Wilhelm Waldeyer** named "chromosomes" in 1888.

For the next hundred years, histochemical and biochemical research into nuclear material has been a scientific focus area peaking in 1953 with Watson & Crick's descriptions of DNA and in 2003 with the complete description of the human genome.

Staining methods to detect nucleic acid

Methyl green-pyronin staining, which dyes DNA green and RNA red, was originally developed by **Ehrlich** in 1898. The method is, however, best-known in the modification described by **Pappenheim** in 1899 (11) and **Unna** in 1902 (12) (Figure 2.1.7).

The method was often used in early histochemical studies of nucleic acid. **Jean Brachet** used it when, in 1939-1940, he described the relationship between cytoplasmic RNA and protein synthesis, which helped to pave the way for what is now called the central dogma of molecular biology:

DNA → RNA → protein (13).

The specificity of the method has occasionally been the object of scepticism due to impurities in the reagents used. In their research in the 1980s, **Hans Lyon** (1932-) and his coworkers demonstrated that the use of pure dyes facilitated specific histochemical investigation of DNA and RNA, and that the DNA reaction could be quantified (14,15).

In 1924, **Robert Feulgen** (1884-1955), working with an assistant named **Rossenbeck**, described a method to detect DNA, which is now called "the Feulgen reaction" (16). This method is probably the 20th century's most important histochemical method to explain biological conditions.

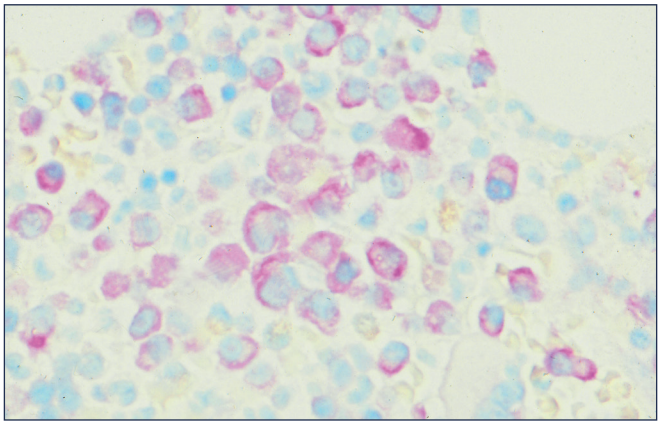


Figure 2.1.7 The Unna-Pappenheim methyl green-pyronin stain for DNA and RNA. Illustrated here from a student laboratory assistant's box (1983). (Tine Meyer)

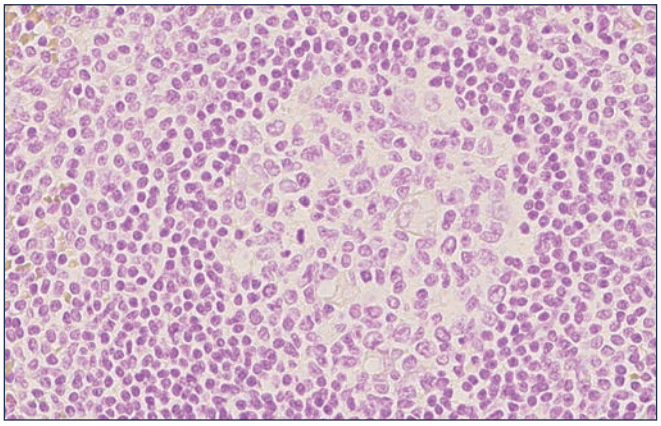


Figure 2.1.8 The Feulgen Stain used to identify DNA. (Ole Nielsen)

Many studies have shown that, under standardised and controlled conditions, the method is specific for DNA and can be cytophotometrically quantified (Figure 2.1.8).

The many factors that the method has helped to clarify include the following: 1: that nuclei in both animals and plants contain DNA, 2: that the striated areas on the chromosomes contain DNA and that this material forms the basis for inherited characteristics, 3: that there is a 1:1 ratio between DNA content in the nuclei and the number of chromosomes, 4: that DNA content is the same in all somatic cells, 5: that DNA content in the nuclei is doubled prior to mitosis.

In connection with research into tumours, the method has been used to determine ploidy in benign and malignant tumours and provides valuable prognostic information.

In 1932, **Lárus Einarson** (Box 2.4) described his gallocyanin-chromalum stain, which can detect DNA and RNA with great specificity.

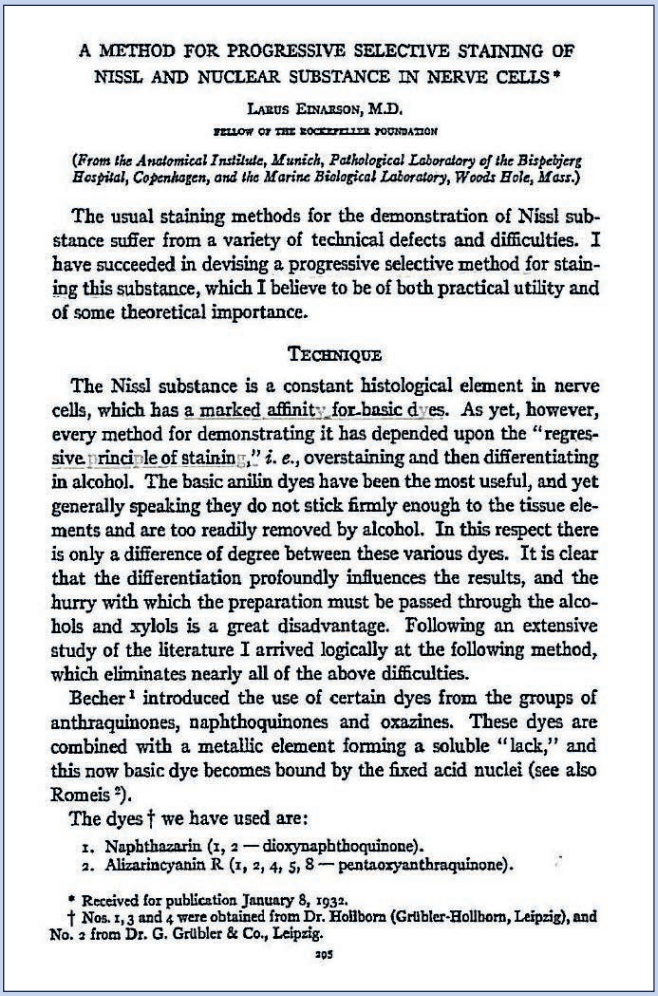
Lárus Einarson (1902-1969)

Figure 1. Lárus Einarson.

Box 2.4

Lárus Einarson (Figure 1) was born in Reykjavik and graduated as a physician there in 1928. During his studies, he became interested in neuroanatomy and, after graduating, he was appointed to work with **F. C. C. Hansen** at the Department of Anatomy in Copenhagen. According to Einarson, Hansen was a very important contact to make. It was probably Hansen's work with haematoxylin staining methods that inspired Einarson to work towards finding a suitable staining to detect tigroid or Nissl substance in nerve cells.

Having worked for **F. C. C. Hansen** for a year, in 1929-1931, Einarson embarked on a study tour, which took him to Munich and later to the US, where he continued to experiment with Nissl staining. In 1932, he published his results in The American Journal of Pathology (1). He discovered that a gallocyanin-chrome alum solution was most suitable and continued to develop his method for some years (Figure 2).



A METHOD FOR PROGRESSIVE SELECTIVE STAINING OF NISSL AND NUCLEAR SUBSTANCE IN NERVE CELLS*

LARUS EINARSON, M.D.

FELLOW OF THE ROCKEFELLER FOUNDATION

(From the Anatomical Institute, Munich, Pathological Laboratory of the Bispebjerg Hospital, Copenhagen, and the Marine Biological Laboratory, Woods Hole, Mass.)

The usual staining methods for the demonstration of Nissl substance suffer from a variety of technical defects and difficulties. I have succeeded in devising a progressive selective method for staining this substance, which I believe to be of both practical utility and of some theoretical importance.

TECHNIQUE

The Nissl substance is a constant histological element in nerve cells, which has a marked affinity for basic dyes. As yet, however, every method for demonstrating it has depended upon the "regressive principle of staining," *i. e.*, overstaining and then differentiating in alcohol. The basic anilin dyes have been the most useful, and yet generally speaking they do not stick firmly enough to the tissue elements and are too readily removed by alcohol. In this respect there is only a difference of degree between these various dyes. It is clear that the differentiation profoundly influences the results, and the hurry with which the preparation must be passed through the alcohols and xylols is a great disadvantage. Following an extensive study of the literature I arrived logically at the following method, which eliminates nearly all of the above difficulties.

Becher[1] introduced the use of certain dyes from the groups of anthraquinones, naphthoquinones and oxazines. These dyes are combined with a metallic element forming a soluble "lack," and this now basic dye becomes bound by the fixed acid nuclei (see also Romeis[2]).

The dyes† we have used are:

1. Naphthazarin (1, 2 — dioxynaphthoquinone).
2. Alizarincyanin R (1, 2, 4, 5, 8 — pentaoxyanthraquinone).

* Received for publication January 8, 1932.

† Nos. 1, 3 and 4 were obtained from Dr. Hollborn (Grübler-Hollborn, Leipzig), and No. 2 from Dr. G. Grübler & Co., Leipzig.

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Figure 2. The title page of Lárus Einarson's paper, in which he describes gallocyanin staining for the first time. (1)

He demonstrated that the Nissl substance varies in different nerve cells. He believed that the variation was cyclic and that the Nissl substance was used if the nerve cells were stimulated to activity and that it regenerated

after a period of rest. When he put forward this theory in 1933, no-one knew the chemical composition of the Nissl substance. Later research revealed that the Nissl substance is made of ribosomal RNA – and that Einarson's theory was correct.

In 1935, Einarson began working as an assistant at the Psychiatric Laboratory in Copenhagen and began to work with the histopathological changes associated with multiple sclerosis, vitamin E deficiency, etc. From 1945, he was a consultant at the Department of Cerebral Pathology in Aarhus.

Einarson became professor of anatomy at Aarhus University in 1936. He and his students produced comprehensive research into nucleic acid metabolism of the nerve cells and other cells in the human organism. Einarson believed that the link between the gallocyanin-chromalum complex and nucleic acid was stoichiometric. With his laboratory manager, he built a cytophotometer to quantify nucleic acid content in the cells (2,3).

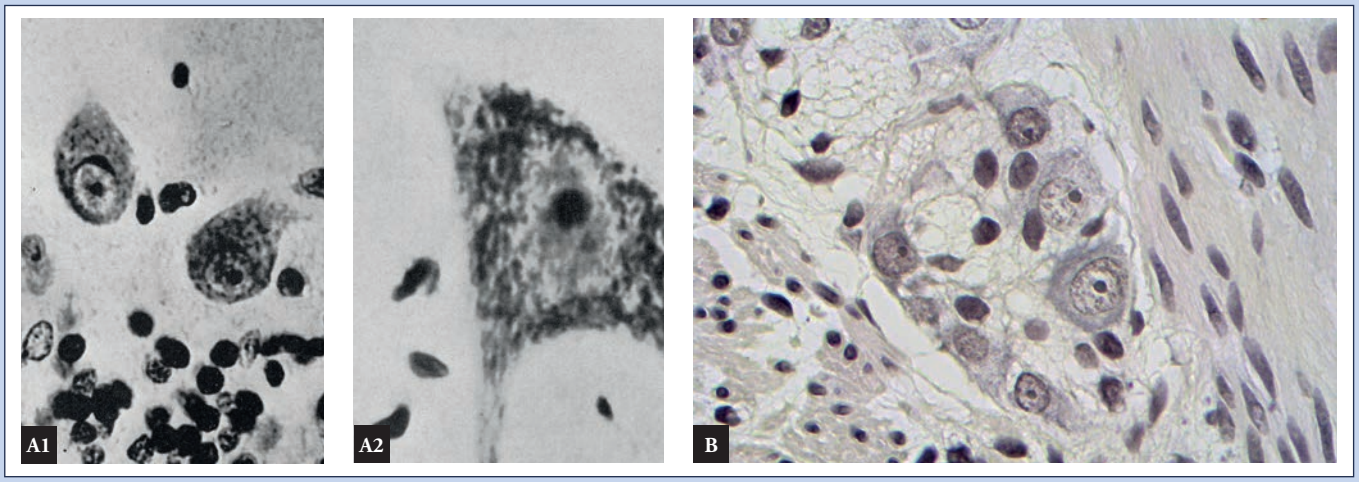

Figure 3. Images of gallocyanin-chromalum staining. **A1**: Purkinje cells from rabbit cerebellum, **A2**: Anterior horn cells from medulla spinalis of a dog. Both are from Einarson's original paper (1). **B**: Staining of ganglia cells in myenteric plexus in the colon. From a student's box used in connection with training to become a hospital laboratory assistant in 1982. (Annelise Olsen)

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2. Einarson L. On the theory of gallocyanin-chromalun staining and its application for quantitative estimation of basophilia; a selective staining of exquisite progressivity. Acta Pathol Microbiol Scand 1951;28:82-102.
3. Einarson L, Hansen E. An apparatus for cytophotometry; determination of the realative degree of basophilia of cell structures with special reference to the nerve cells. Acta Psychiatr Neurol Scand Supp. 1956;108:151-68.

Carbohydrate staining methods

To complete our review of the development of histochemical staining methods until the mid-1900s, we mention two methods, both of which were described during this period and are used as mucin stains: PAS and Alcian blue.

The PAS (Periodic acid Schiff) reaction was described among others by McManus in 1946 (17). It has since become the preferred staining method used to detect glycogen and neutral glycoproteins (Figure 2.1.9).

Alcian blue is an example of a dye, which was initially produced for use in the textile dyeing industry and only later used in histochemical staining. The use of alcian blue in histology was described by **Steedman** in 1950 (18) and has since found widespread use in detecting carboxylated and sulphated mucosubstances (Figures 2.1.10 and 2.1.11).

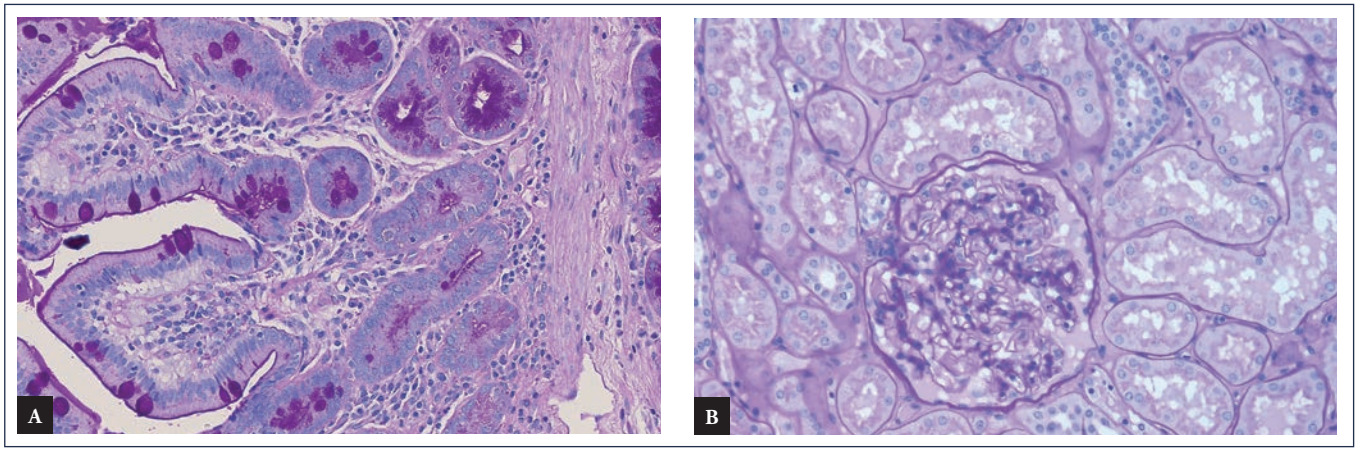

Figure 2.1.9 PAS stain used to detect **A** neutral mucines in duodenal mucous membrane and **B** glycoproteins in basement membranes and the mesangial area of the renal glomerulus. (Ole Nielsen)

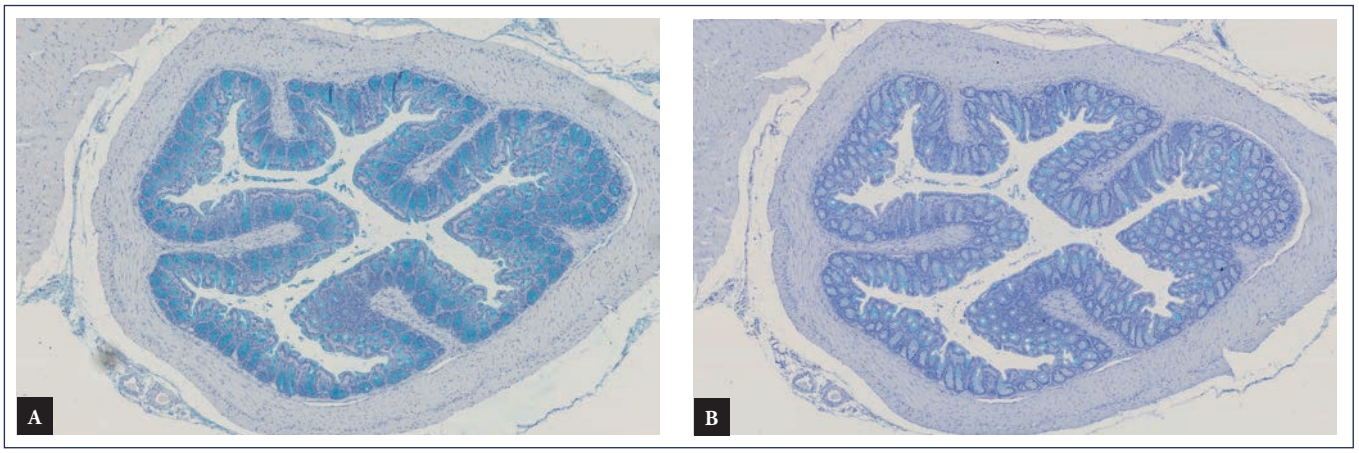

Figure 2.1.10. Alcian blue staining of rectal mucous membrane of a mouse. **A**: Section stained using pH 2.7 to detect carboxylated sialomucins and sulfomucins. **B**: pH 1 stain, which stains sulfomucins only. (Ole Nielsen)

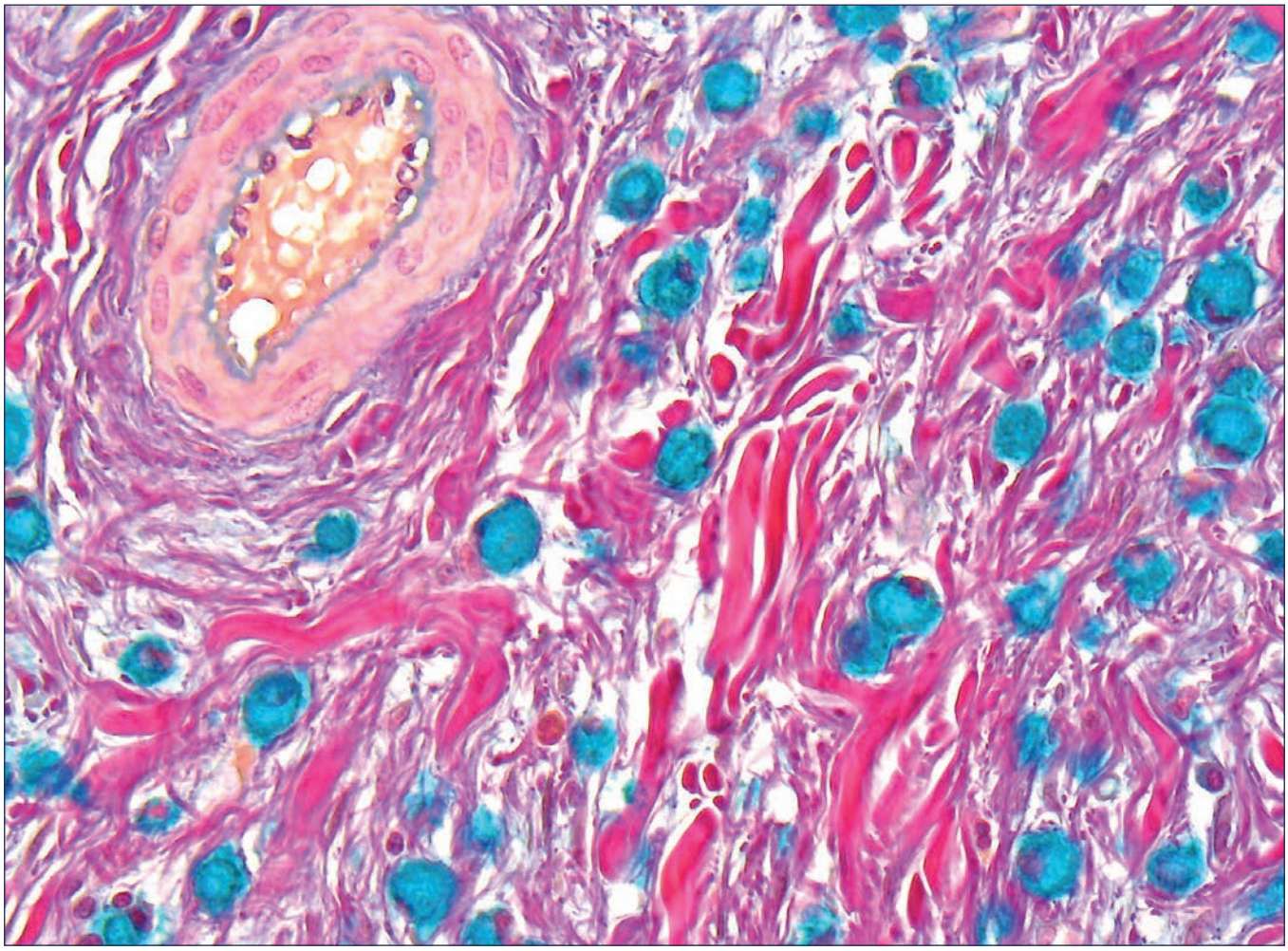

Figure 2.1.11. Eskelund Staining

Viggo Eskelund (1893-1972) was an associate professor at the Department of Pathological Anatomy at the University of Copenhagen from 1944. Staining was his special interest. In 1957 (19), he published a pentachrome stain. This was a variant of alcian blue staining, which e.g. stains mucosubstances blue-green, muscle tissue yellow, collagen red and elastin violet. The image shows a staining of a sigillocellular carcinoma, in which mucins in the cytoplasm of the tumour cells are stained green-blue, whereas the surrounding connective tissue is red. At top left, an arteriole with yellow-stained musculature and violet elastin. (Ole Nielsen)

2.2: ENZYME HISTOCHEMISTRY

The first report on enzyme histochemical staining was probably **E. Klebs'** 1868 description of blue-stained granulocytes with guaiacol, a reaction caused by peroxidase (20).

As chemical and biochemical research in the early 1900s had gradually mapped the significant role played by enzymes in cell and tissue metabolism, there was a natural desire to localise and describe the structural basis of cellular enzyme activity.

One important contribution to this description was research carried out from 1930 at the Carlsberg Laboratory by a close-knit team comprising **Kaj Linderstrøm-Lang**, **Heinz Holter** (Box 2.5) and their staff. Their results were described in a series of publications *Bidrag til den enzymatiske Histokemi (Studies on enzyme histochemistry)*. Linderstrøm-Lang and Holter discovered and developed micromethods to detect enzyme activity in quantities that were a thousand times smaller than those required in the methods generally used at that time. In some cases, enzyme activity could be measured in a single cell. In 1934, papers about the distribution of cells that produce pepsin, acid, esterase and peptidase in the gastric mucosa of pigs were published (21). They were no *in situ* methods as enzyme assays and histomorphological analyses were carried out on neighbouring sections cut on cryostat. The research continued for several years. In the 1950s the scientists helped to show how dehydrogenase was related to the mitochondria in organelle fractions, while hydrolytical enzymes, such as acid phosphatase and cathepsin, were localised in lysosomes.

In 1939, **Gomori** (22) and **Takamatsu** (23) independently described the first *in situ* reaction to demonstrate the presence of alkaline phosphatase. Then, in 1944, the description of azo-dye reactions marked the advent of hydrolase histochemistry. From the mid-1950s, new methods were developed very quickly.

Kaj Ulrik Linderstrøm-Lang (1896-1959)

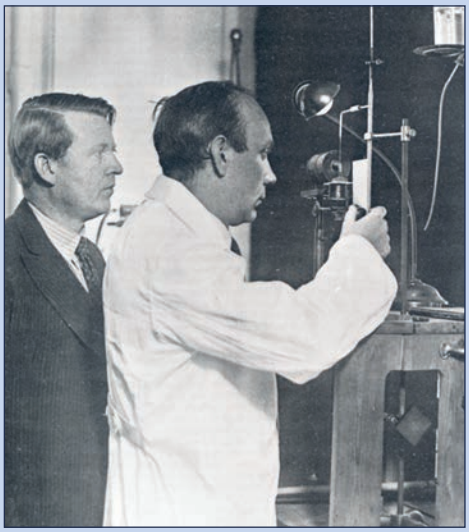

Figure 1. Linderstrøm-Lang and Holter photographed in 1936, when their enzyme histochemical research was well under way. (Ugejournalen, 19 April 1936).

Box 2.5

Kaj Ulrik Linderstrøm-Lang qualified as a chemical engineer in 1919 and later that year he was hired to work with **S.P.L. Sørensen** (1868-1939) at the Carlsberg Laboratory. Sørensen led the chemistry department and is famous for having introduced the pH scale. Lang remained an employee of the Carlsberg Laboratory for the rest of his life. From 1938, he became professor and leader of the chemistry department. He conducted a series of ground-breaking physical-chemical investigations into proteins and enzymes. He is recognised for having described the primary, secondary and tertiary protein structures.

A large protein science research centre at The University of Copenhagen is named after him: http://www1.bio.ku.dk/english/research/bms/research/llc/

Heinz Holter (1904-1993)

Heinz Holter was born in the erstwhile Austro-Hungarian Empire. He read chemistry at the university in Vienna and received his doctorate in 1928. At this time, he began to show some interest in biochemistry and in 1930 he came to the Carlsberg Laboratory on a 12-month Rockefeller scholarship. He remained in Denmark and worked at the Carlsberg Laboratory for the rest of his life. He became a Danish citizen in 1939, leader of the cytochemical department in 1944 and was professor and leader of the physiological department from 1956 (Figure 1).

On his arrival in Denmark in 1930, Holter suggested to Lang that they localise the enzymes in tissue and cells. Lang was eager to accept the challenge. This was the start of a 20-year partnership, which produced a large series of publications entitled *Bidrag til den enzymatiske Histokemi (Studies on enzyme histochemistry)*.

These works included not only significant clarifications regarding enzyme histochemical conditions in plants and animals, but also protocols for a number of technical inventions for laboratory use. In addition to the invention of brilliant micromethods that detected enzyme activity even to single-cell level (1), the team also built a cryostat with an anti-roll plate (Figure 2), which was the prototype for cryostats subsequently released for commercial production. The constriction (Carlsberg) pipette (Figure 3) was also invented during this period. The Carlsberg pipette was widely used in laboratories around the world until it was superseded by disposable pipettes.

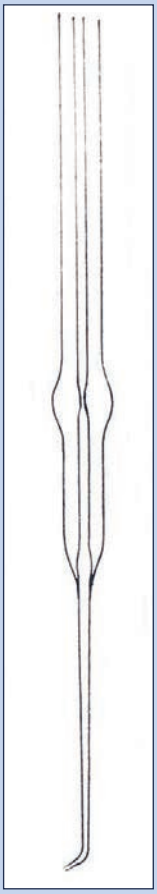

Figure 3. The Carlsberg or constriction pipette.

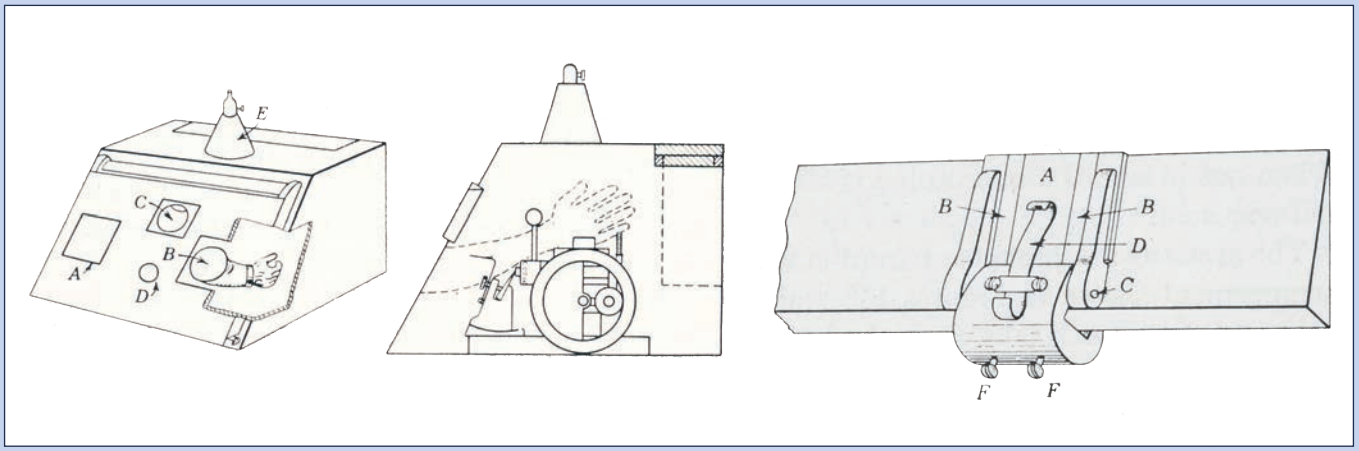

Figure 2. The Lang-Mogensen cryostat with anti-roll plate. (2)

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In 1953, in the first edition of **A.G. Everson Pearse's** *Histochemistry, theoretical and applied*, he wrote that, until just a few years before its publication, there were methods available to detect only 2-3 enzymes but that this number had now increased to about 18. Five years later, this number was 45 and after a further 8 years, 75 enzymes could be detected and a further 30 could be localised by using simple adaptations of existing techniques.

Enzyme histochemical methods were used extensively in research, also at the ultrastructural level. In Denmark, there were several dissertations within embryology (see 3.1), haematology (24) and mamma pathology (25), which used enzyme histochemical methods. In diagnostic histopathology, these methods have been used in particular within haematology and muscle pathology (Figure 2.2.1).

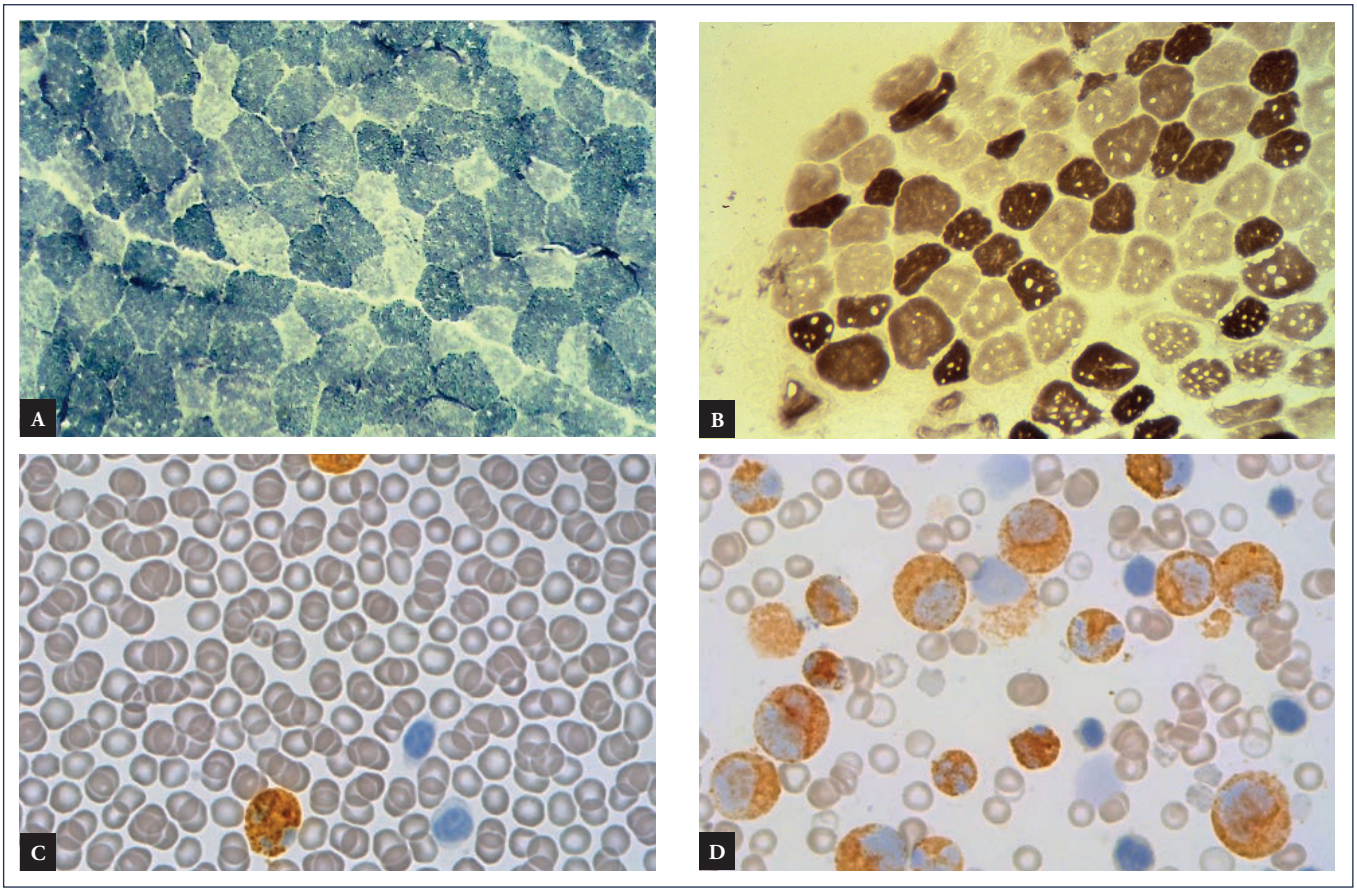

Figure 2.2.1 Enzyme stainings. **A+B**: Staining of section of striated musculature. **A**: NADH-dehydrogenase and **B**: ATPase. **C+D**: Peroxidase staining. **C**: Normal blood with a positive reaction for neutrophile granulocytes and negative reaction for lymphocytes. **D**: Bone marrow cells with positive reaction in myeloid cells. (Henrik Daa Schrøder, Ole Nielsen, Annelise Olsen)

2.3: IMMUNOHISTOCHEMISTRY

The idea of utilizing the antibodies' incredible specificity vis-à-vis their respective antigens and – by means of appropriately labelling the antibodies – exploiting them as colour reagents, is not new. In 1930, **L. Reiner** demonstrated that it was possible to link diazotized atoxyl (a sodium salt of aminophenylarsonic acid) to antibodies against pneumococci and thus to produce an "antibody dye", which maintained its immunoreactivity (26). This was confirmed in 1934 by **John Marrack** (27). In 1941, **Albert H. Coons** and his staff picked up on the idea (28) and tested it on histological material. The staining reaction they achieved was too weak to be useful as a histochemical method.

2.3.1: Immunofluorescence methods

Coons went on instead to use a link between blue fluorescent β-anthryl isocyanate and achieved useful results using ultraviolet light. To achieve a greater contrast with the autofluorescence of the tissue, in 1942 he switched to using the green fluorescent fluorescein-4-isocyanate, which produced a satisfactory result (29). In the wake of its humble infancy in the 1940s, where the difficulties were compounded by the fact that most scientists had to make their own antibodies and conjugates, the method became much more widely used from the mid-1950s. Contributory factors included the availability of new, improved and (gradually) more commercially accessible conjugates, including FITC (fluorescein isothiocyanate), TRITC (tetramethylrhodamine isothiocyanate) and Texas Red (sulforhodamine 101) (Figure 2.3.1.1) and the development of fluorescence microscopes with interference filters (see 3.3.1).

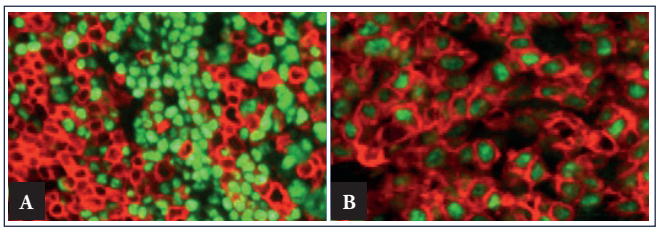

Figure 2.3.1.1. Immunofluorescence staining used as double-staining. **A**: Section of reactive lymph node. **B**: Section of lymph node in a patient with B-CLL. The sections are stained for PAX-5 (green), with Alexa Fluor 488 (FITC), and for CD5 (red) with Alexa Fluor 594 (Texas Red). (Michael Bzorek)

This method was and still is widely used in research and diagnostics on tissue and cell cultures. Its innumerable uses include the investigation of microorganisms, hormones in the pituitary gland, pancreas, gastrointestinal tract and thyroid gland, enzymes and blood group antigens in tissue (see 3.3.2) and plasmaproteins, including immunoglobulins under physiological and pathological conditions in particular. Clinicians and diagnosticians use the methods to detect autoantibodies and in differential diagnostics of glomerulonephritis and bullous skin diseases (see 3.3.3 and 3.3.4) and, in connection with flow cytometry for differential diagnostics of haematological disorders.

2.3.2: Enzyme immunohistochemical methods

A new era of immunohistochemistry dawned in 1966, when **Nakane & Pierce** described the use of enzyme-labelled antibodies to localise antigens in tissue (30). Among the enzymes then tested, in particular peroxidase (extracted from horseradish) and, to a lesser extent, alkaline phosphatase were used. Enzyme-labelled antibodies offer a number of advantages in immunohistochemical assays. The reaction product, which is permanent, can be examined in an ordinary brightfield microscope and, like the "classical" staining methods, allows for simultaneous assessment of tissue morphology. Moreover, because the reactive product is electron-dense, the method can be used in connection with electron microscopy, where ferritin and, especially, gold are used.

Since 1966, the enzyme immunohistochemical methods have been modified with a view to improving their sensitivity (Figure 2.3.2.1).

One important parameter in immunohistochemistry is **tissue treatment**, which should, if possible, facilitate retention of the antigen reactivity of the structures and substances that the scientist wishes to stain. In the early days, a cryosection was taken and then fixated using acetone or ethanol as this was regarded as the least damaging method. A Norwegian, **Per Brandtzaeg** developed a method using 75% ethanol fixative and subsequent paraffin-embedding to detect immunoglobulins.

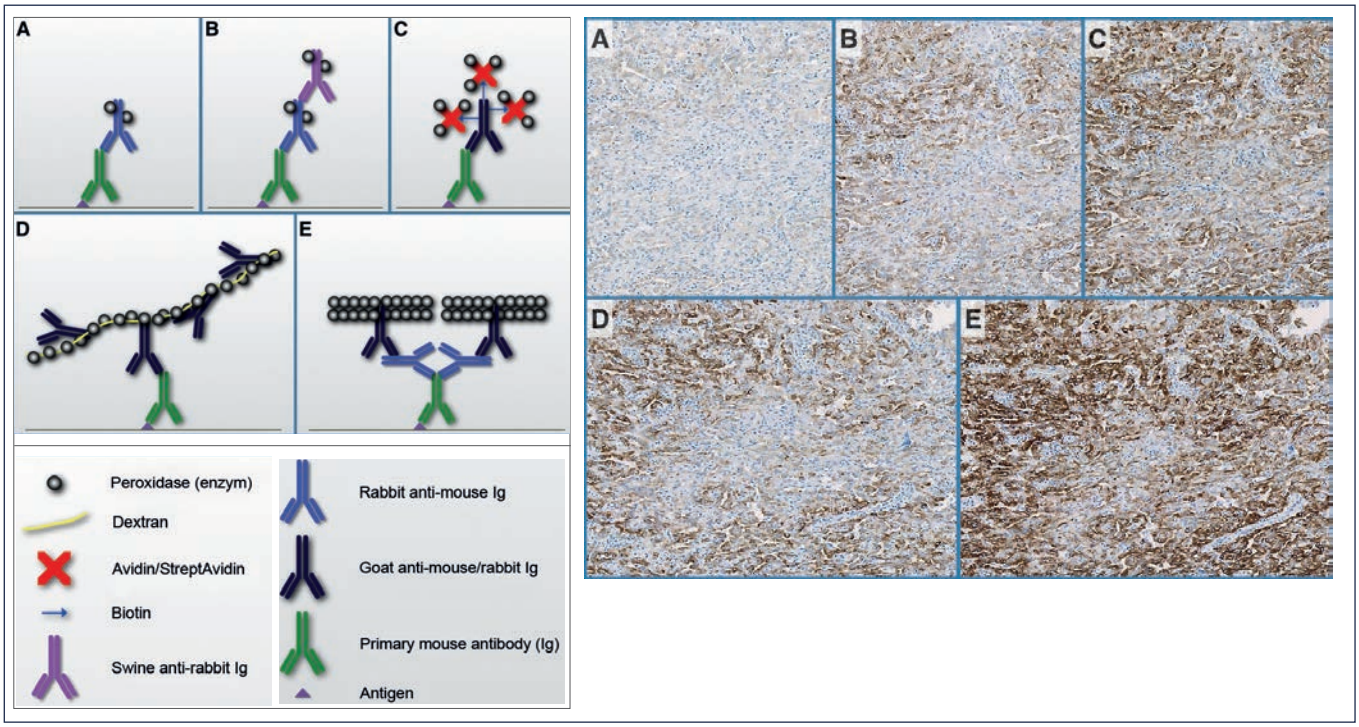

Figure 2.3.2.1. The development of enzyme immunohistochemical investigation systems aimed to achieve as large a number of signal enzymes per primary antibody as possible. **A-E** (left) show the principles used in a selection of systems introduced since the early 1970s. **A**: Two-step technique, **B**: Three-step technique, **C**: Labelled streptavidin biotin (LSAB) method, **D**: Two-step polymer method (EnVision™), **E**: PowerVision™ (three-step polymer technique). On the right, the corresponding stain results for renal cell carcinoma stained for cytokeratin 19, in which all the method parameters including primary antibody concentration are constant, while the investigation systems vary. (Ole Nielsen)

In 1974, **Clive R. Taylor** and **John Burns** announced (31) that it was possible to stain plasma cells and other cells containing immunoglobulins in formalin-fixed and paraffin-embedded tissue. This was a real breakthrough for the use of these methods in diagnostic histopathology. It turned out, however, that not all antigens withstand this treatment with equal success. In 1975, **Huang** (32) found that treating tissue with trypsin could repair much of the damage – in some antigens at least. Later (in 1991), **Shi** et al. (33) described a method, in which pre-treating paraffin sections in suitable buffers at temperatures above 100°C could revive antigen reactivity in many antigens. This antigen retrieval method is now a standard method (Figure 2.3.2.2).

After 1974, these methods rapidly became popular in diagnostic histopathology. A Danish expert in proteins, **Niels Harboe** (Box 2.6) had set up a company, DAKOPATTS, just a few years earlier. Until then, the supply of commercially accessible antibodies and conjugates was limited. With DAKOPATTS' help, these became significantly more readily available and several foreign companies joined the market. A further advance in the supply of antibodies came with the launch of monoclonal antibodies in the 1980s.

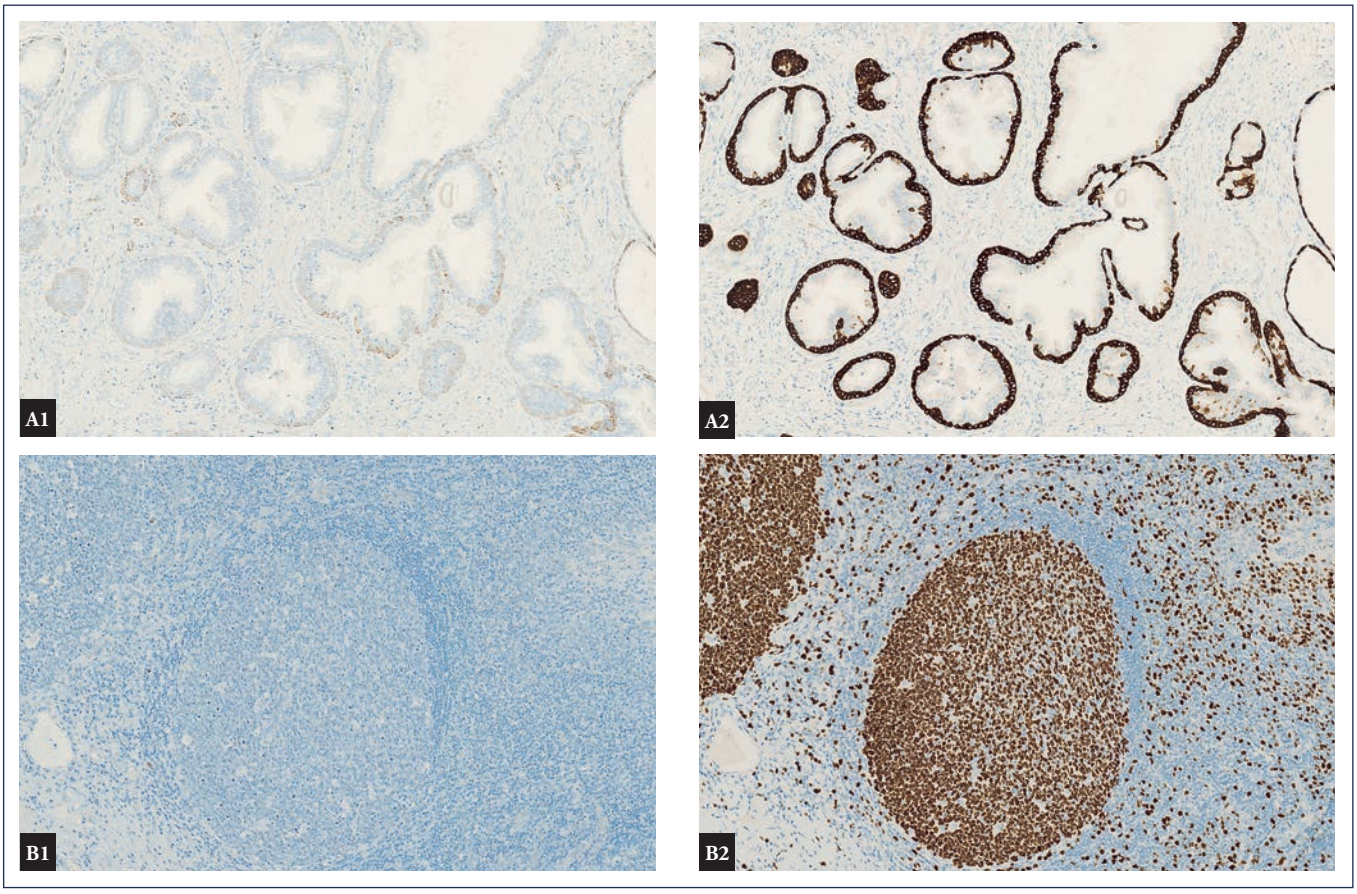

Figure 2.3.2.2. Antigen retrieval. **A**: Immunohistochemical staining of prostate tissue stained for CK14 without (A1) and with (A2) retrieval. **B**: Staining of germinal centre of lymph node stained for Ki-67 without (B1) and with (B2) retrieval. (Ole Nielsen)

The colossal supply of antibodies meant, however, that, from time to time, scientists found the reagents were ineffective. There was an obvious need for systematic quality control. Significant Danish contributions to the introduction of systematic quality control are described in Chapter 4.

The increase in the number of immunohistochemical stainings made at pathology units makes automation a necessity. There are several commercial automated systems on the market.

Niels Mathias Gunnersen Harboe (1918-2006)

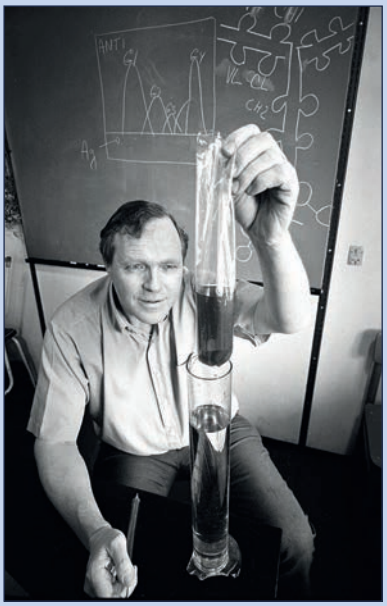

Figure 1. Niels Harboe in the laboratory in 1975. Behind him, a blackboard outline of a crossed immunoelectrophoresis and the general basic structure of an immunoglobulin molecule. (Morten Langkilde - POLFOTO)

Box 2.6

Having graduated from The University of Copenhagen as a Doctor of Medicine in 1945, **Niels Mathias Gunnersen Harboe** (Figure 1) set off to stay from 1946-1949, at what he described as "The Mecca of Proteinchemistry": He attended The Institute of Physical Chemistry and Biochemistry at Uppsala University in Sweden. Here he was taught protein analysis and protein separation methods by a fellow Dane, **Kai O. Pedersen**. The institute was headed by Nobel Prize Laureate, **The Svedberg** and by **Arne Tiselius**, who received the Nobel Prize in 1948, while Harboe still worked at the institute. Back home in Denmark, Harboe founded "Elektroforeselaboratoriet" (The Electrophoresis Laboratory) at The University of Copenhagen in 1949. The laboratory was later renamed "Proteinlaboratoriet" (The Protein Laboratory). Harboe was head of the laboratory until 1979.

In addition to extensive research activities, which over the years resulted in a large number of doctoral theses, the laboratory earned its keep for more than 20 years by performing analyses of plasma and urine proteins for Danish hospitals. At that time, these analyses required a wealth of expertise and were costly in terms of both time and equipment.

In 1965 and 1966, a Swedish scientist, **C.-B. Laurell** introduced crossed immunoelectrophoresis and rocket immunoelectrophoresis. These techniques represented a quantum leap in protein research and clinical immunochemistry as they allowed rapid and simple large-scale analysis and quantitative determination of individual proteins in complex protein solutions. The problem was that these techniques required many specific, high-quality antibodies and the market was only to a limited extent equipped to supply these. Moreover, the antibodies that were available were prohibitively expensive, seen in the light of a university laboratory's budget. Niels Harboe would have preferred that a production of antibodies was set up at The University of Copenhagen. The university was, however, not at all interested. Therefore Niels Harboe saw no other solution than, in cooperation with his brother, **Gunner Harboe**, to start a production of antibodies in rabbits on Gunner's farm. Their company, DAKOPATTS, was founded in 1966. The last seven letters of the company name are the initials of Niels Harboe's mentors.

Based on Niels Harboe's skillfulness and protein-chemical expertise, monospecific polyclonal rabbit antibodies with uniform titer from batch to batch now became available on the market for the first time, and research scientists at Proteinlaboratoriet and at many other laboratories were provided with a generous supply of inexpensive or even free antibodies. The company grew, changed its name to Dako and became a global enterprise, its focus gradually shifting to the production of antibodies and other reagents for the immunohistochemical field. Dako, however, continues to develop and produce antibodies for clinical immunochemistry and flow cytometry and also makes reagents and kits for molecular pathology.

In many ways, Dako's product launches both reflect and have influenced the developments within immunohistochemistry: In 1971, the first fluorochrome-conjugated antibodies appeared, followed by enzyme conjugates in 1972. In 1974 Dako developed the new, sensitive peroxidase-anti-peroxidase-visualization reagent (PAP), which was originally described by **Sternberger** in 1970 (1). Using this Dako reagent, **Clive R.Taylor** in Oxford succeeded in detecting immunoglobulins in archival formalin-fixed tissue more convincingly than was previously thought possible (2). In the wake of Taylor's success, interest in and sales of antibodies for use in enzyme immunohistochemistry increased dramatically. PAP was the predominant visualization reagent on the market for about 12 years, after which it was supplemented and gradually replaced by several Dako visualization reagents that were even more sensitive and each presented benefits of its own.

As indicators for a new and ground-breaking development in antibody production, Dako's first ten monoclonal mouse antibodies were released onto the market in 1983. The product portfolio was expanded with an instrument, which offered semi-automated immunostaining in 1995. A peak (as far as instruments are concerned) was reached in 2014 when Dako introduced a fully automated instrument, which, using validated protocols, can conduct any immunohistochemical process, including deparaffinization, immunostaining and counterstaining, on up to 60 tissue sections within 2½ hours.

In 1973, Dako produced antibodies against 23 specific antigens/targets. In 2015, this figure had increased to 225, i.e. 44 polyclonal and 181 monoclonal primary antibodies for immunohistochemistry. The 2015 Dako catalogue featured a total of 810 products, including 11 instruments, for use in pathology laboratories.

Dako has been in foreign hands since 2007 and was sold to Agilent Technologies for DKK 12.8 billion in 2012.

In 1989, Niels Harboe was made an Honorary Doctor of Medicine at The University of Copenhagen.

Agnete Ingild

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2.4: IN SITU HYBRIDIZATION

Our historical review of histochemical methods would not be complete without a description of the latest, but probably not the last, method to be developed: *In situ* hybridization, which was first described in 1969 (34,35).

This method offers localisation and visualisation of limited areas of DNA and RNA molecules in tissue sections, cytological material or parts thereof, i.e. chromosomes, using complementary nucleotide sequences (probes) directed at larger or smaller parts of DNA or RNA single-string nucleotide sequences. The probes are tagged with markers and paired with their respective complementary partners on the nucleic acid strings (hybridised), after which the reaction can be observed. If the probes are tagged with fluorescent dyes (FISH = fluorescence *in situ* hybridization), The reaction can be seen with a fluorescence microscope. If the probe is tagged so that the final reaction is stained with a chromogen (CISH = chromogenic *in situ* hybridization), the reaction can be seen with an ordinary brightfield microscope, with all the practical benefits this has to offer (mentioned earlier in the chapter on immunohistochemistry). Another variant, which uses silver, is called silver-enhanced ISH (SISH). CISH and SISH are both brightfield *in situ* hybridization methods (BRISH) (Figure 2.4.1).

These methods have spread to many fields of research and diagnostics within cell biology, medicine and pathology. For example, they can be used to localise specific genes or the absence thereof in chromosomes, to detect viruses and oncogens in cells and tissue sections, and to detect different types of RNA (mRNA, IncRNA and miRNA) in cells, circulating tumour cells and tissue sections. Within cancer research and diagnostics, these methods are increasingly used to assess prognosis and disease remission. Some of the newer applications are described in Chapter 3.5.

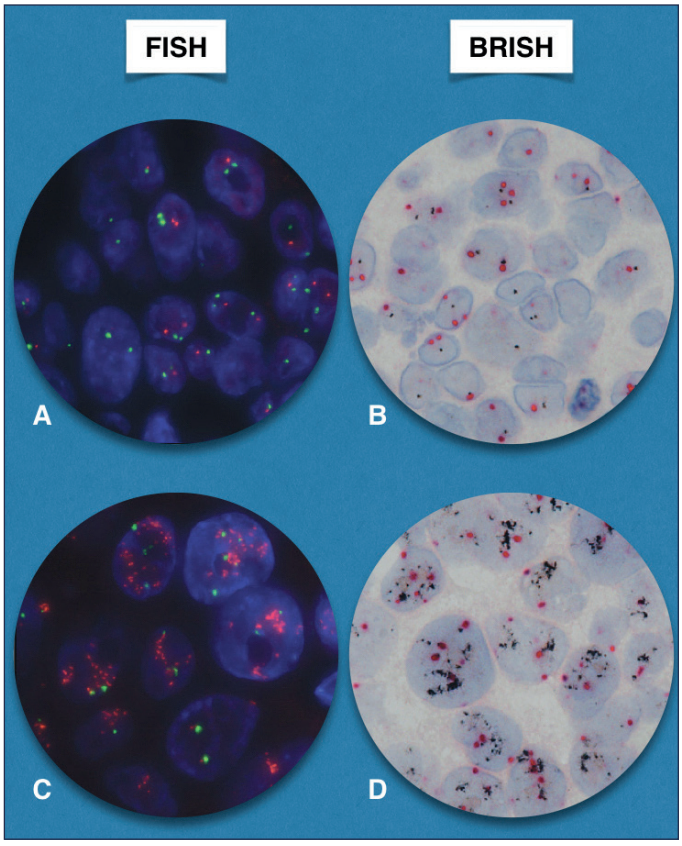

Figure 2.4.1 Examples of fluorescence *in situ* hybridization (FISH) (**A** and **C**) and brightfield *in situ* hybridization (BRISH) (**B** and **D**) of HER2 and CEN-17 detection in formalin-fixed paraffin-embedded tumour xenografts. MCF7 xenograft shows non-amplified HER2 gene and chromosome 17 polysomy (**A** and **B**). BT-474 xenograft shows amplified HER2 gene and chromosome 17 polysomy (**C** and **D**). The BRISH technique is used here. We see the HER2 gene signal as small, black spots of silver, while CEN-17 is seen as slightly larger red signals. The HER2 FISH signal is orange-red, while CEN-17 is green. (Ventana Medical Systems)

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CHAPTER 3: HISTOCHEMICAL METHODS IN RESEARCH AND DIAGNOSTICS

3.1: CYTO-AND HISTOCHEMICAL RESEARCH AT THE FORMER INSTITUTE OF MEDICAL ANATOMY AT THE UNIVERSITY OF COPENHAGEN - *by Kjeld Møllgård and Ole William Petersen*

3.1.1: Helge Andersen, pioneer of histochemistry and human embryologist, and the Cyto- and Histochemistry Laboratory (CHL)

Following F.C.C. Hansen's comprehensive body of work on haematoxylin staining at the turn of the century (see box 2.2), with a single exception (**Harald Okkels** (1898-1970)), there was only relatively modest activity within histochemistry-related research at the Institute of Medical Anatomy in Copenhagen for almost the next fifty years.

This situation changed radically when, having become a doctor of medicine in 1957, **Helge Andersen** (1929-1995) (Box 3.1) was recruited to work as scientific assistant in the department in 1959. In the following year, he set up and subsequently managed the *Cyto- og Histokemisk Laboratorium (Laboratory of Cyto- and Histochemistry)* (CHL).

Within the field of histochemistry, he was self-taught and received no real support or training from older scientists or colleagues. He was without doubt the pioneer and founder of modern histochemistry in Denmark. From the division of the institute into three parts in 1965 until he retired in 1983, Helge Andersen was lecturer and departmental manager at Medical Anatomical Institute A. His main field of research was human embryology where he initially focused on the histochemistry of joint and bone development, which resulted in a thesis, *Histochemical investigations into osteoarticular development in human foetuses in the first half of the prenatal period*, which he submitted to The University of Copenhagen in 1968 and defended in 1970 (1).

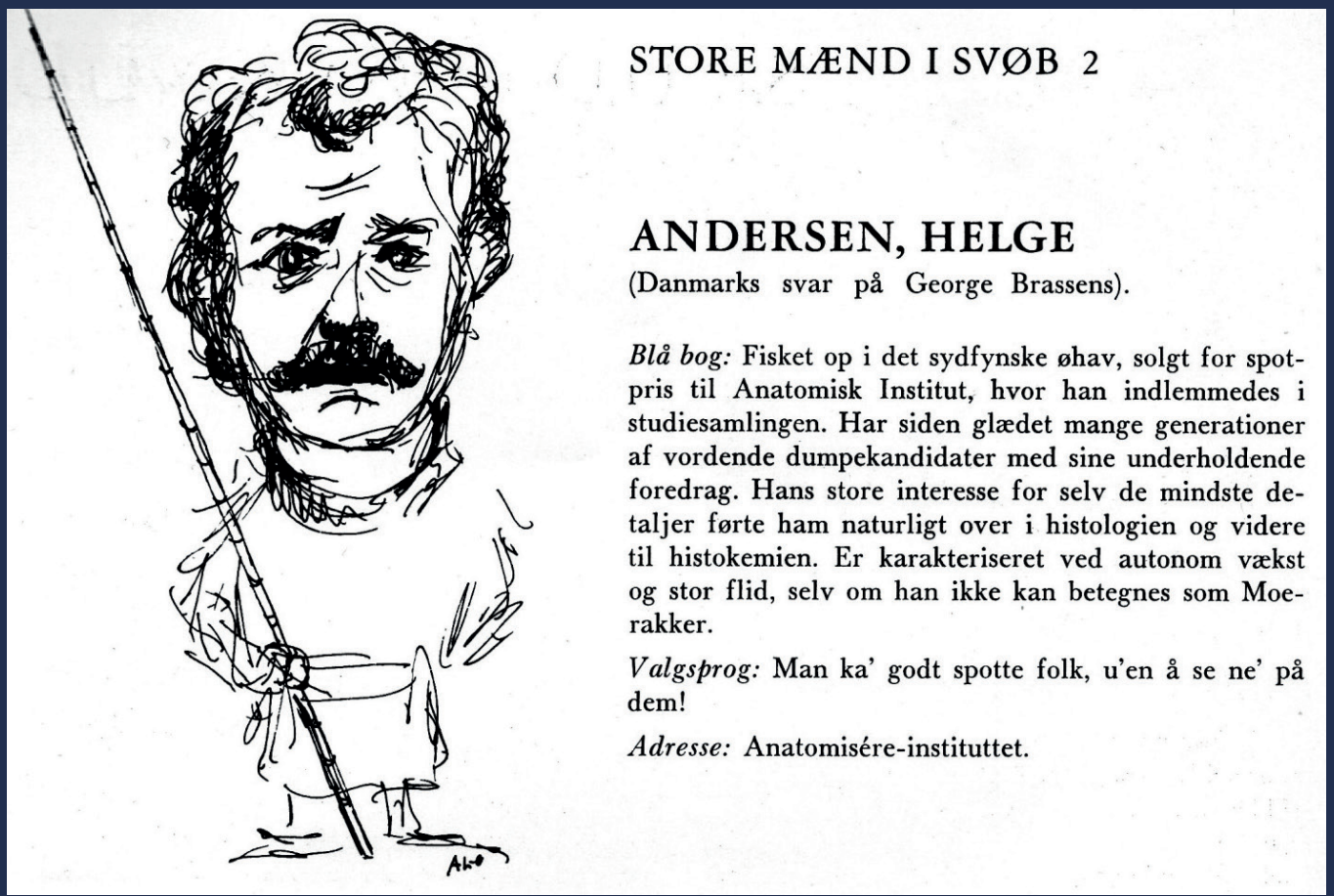
Box 3.1

Eye witness report from the Sorcerer's workshop

Nørre Allé in 1968 by *Kjeld Møllgård*

In the summer of 1968, straight after my final exams, I was hired to work as a research assistant at the Medical Anatomical Institute, following 4 years of student employment at the institute – as a demonstrator and anatomical instructor. Helge Andersen had agreed to teach me histochemistry and embryology. Unlike self-taught Helge, I was an apprentice in the sorcerer's workshop. His laboratories were lined with shelf upon shelf of bottles filled with alkaline substances and, of course, with sulphuric acid, nitric acid, hydrochloric acid, even picric acid – alongside the indispensable glacial acetic acid. There were also many different metal containers with all kinds of powdered dyes, e.g. 3 kilogrammes of Alcian blue from G. T. Gurr next to Astrablau and the mandatory toluidine blue. There was also every imaginable chemical, innumerable enzymes (in particular a great many phosphatases, peptidases and dehydrogenases), rare minerals and plenty of cyano compounds and other poisons, most often locked away in poison cabinets. This was Helge's domain, (affectionately portrayed in the student's yearbook "Bugpressen" (Belly Press) (Figure 1)), wearing a black polo neck sweater and brown twill jacket, surrounded by bubbling nitrogen- and CO_2 containers smoking his pipe as he received fresh abortion material from one of the gynaecological units in Greater Copenhagen. The laboratory's histo-laboratory assistants fetched the material by car.

Highly motivated research scientists and laboratory assistants at the institute's cyto- and histochemical department worked in a very creative environment. Everyone was on their toes when fresh material arrived. Telephones rang and researchers from near and far turned up to get their share of the "cake", i.e. material they could use in their specific field of research.



STORE MÆND I SVØB 2

ANDERSEN, HELGE

(Danmarks svar på George Brassens).

Blå bog: Fisket op i det sydfynske øhav, solgt for spotpris til Anatomisk Institut, hvor han indlemmedes i studiesamlingen. Har siden glædet mange generationer af vordende dumpekandidater med sine underholdende foredrag. Hans store interesse for selv de mindste detaljer førte ham naturligt over i histologien og videre til histokemien. Er karakteriseret ved autonom vækst og stor flid, selv om han ikke kan betegnes som Moerakker.

Valgsprog: Man ka' godt spotte folk, u'en å se ne' på dem!

Adresse: Anatomisére-instituttet.

Figure 1. From "Bugpressen, Våren 1965" (Belly Press.Spring 1965). The cartoon refers to Helge's roots. He was the son of a sea captain from Ærø (an island just south of Funen). The text makes reference to his characteristic South Funen accent.

From Nørre Allé to the Panum Institute by *Ole William Petersen*

The idyll that characterized the Cyto- and Histochemical Laboratory described above was in many ways interrupted when the anatomical departments moved from Universitetsparken and the Rådmandsgade complex to the Panum Institute in 1979. Helge never really embraced Panum with its brutal concrete design expression. The department was split up. Apart from Helge himself, only Charly Garbarsch and Poul Erik Høyer practised cytochemistry in its purest form.

In 1981, still a medical student, it was my pleasure to start work as an assistant tutor in histology. Helge Andersen was tutor. I can confirm that Helge's approach to lecturing was narrative. He possessed an unique talent for the narrative. Few could make their point in chalk on the blackboard as he. He spellbound his listener. I was indeed honoured to be assistant tutor and I learned many pedagogical tricks, of which I have since been able to reap full benefit in my own work. Helge most likely found it worthwhile to invest in me. Thus, I was invited up to the "holy of holies" in building 18 on the 4th floor. With great expectations, I accepted gladly in the summer of 1981. I soon realised that Helge's insight into human histology was only one facet of his immense repertoire of knowledge, and that his passion was in fact histochemical methodology and, not least, the potential pitfalls. In this way I developed a critical eye for the histochemical methods, which I have never forgotten and which, as it turned out, seemed to be characteristic of histo- and cytochemists in general. Of course it was also the source of numerous scientific discussions at our end of the department. I arrived when brilliant microscopists – working in real time in a 37 °C heated room or at 4 °C in the cold room – had to compete with the blessings of the microspectrophotometer. Relative readouts like one of two pluses and time until appearance of reaction product were up against accurate densitometry. I learned both methods and even now I still believe that to achieve the best results you need to appreciate both the limitations of the instruments and to include sensible controls. Since most of the time I was the only student at CHL, I got more than enough advice and it was not necessarily unequivocal. Of course it demanded a lot of diplomacy and I learned to extract the best practice.

When working in the lab with Helge, for example, I was strictly advised against using these "next generation" plastic pipettes. Helge believed that plastic and cytochemistry simply did not mix. However, I could see from the shelves that glass equipment was gradually being replaced by disposable products. Experiments with Poul Erik Høyer were more liberal in that respect.

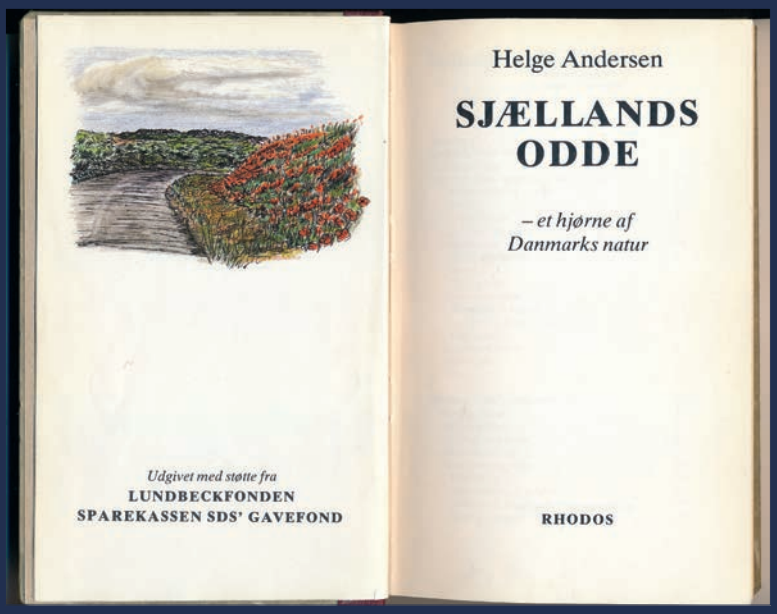


Figure 2.

You may argue that I tend to romanticise the time I spent at 18.4 while I was a student. I can't help it. I was very privileged to serve as an apprentice among so many talented and generous people and, not least, Helge Andersen. I was also honoured to receive a copy of Helge's book on the flora and fauna of the Odsherred peninsula as a gift when celebrating my new title (dr.med.) in 1990. "Congratulations on your five letters and two full stops," he wrote. His book is written as meticulous as his histo- and cytochemical observations (Figure 2).

From the early 1960s, inspired by Helge's enthusiasm and encyclopaedic knowledge, there were other people at CHL working with histochemistry and foetal development. **M.E. Matthiessen**, who was both a qualified dentist and physician, made an important contribution via his studies of foetal dental development. He started working as a volunteer at CHL in 1962. He was departmental manager and associated professor from 1967. A total of nine articles formed the basis for his dissertation, which he defended in 1973 (2). **Charly Garbarsch** was associate professor at the institute from 1967. He also started histochemical investigations of human foetuses, in particular on intestinal development (3). He later turned into experimental research into the aorta wall in rabbits, which was later the subject of his thesis. Working with Ib Lorenzen and his team, Garbarsch continued research with connective tissue at the institute.

In the early 1960s, **Niels Ehlers**, later professor of ophthalmology at Aarhus University, described *Histokemien og udviklingen af humane øjenlåg (Histochemistry and the development of the human eyelid)* in partnership with Helge Andersen and M.E. Matthiessen. His later works included a body of histochemical studies focusing on corneal development (4).

Kjeld Møllgård was recruited in 1968 (see below).

When **Poul Erik Høyer** arrived in the early 1970s, quantitative histochemistry, research into steroid dehydrogenase (5,6) and gonad development took off in earnest, the latter in a fruitful collaboration with Anne Grete Byskov from the Laboratory of Reproductive Biology at Rigshospitalet (RH) in Copenhagen. Høyer became the laboratory's histochemist *par excellence*. Throughout his career, he worked to further develop his subject and, when Helge Andersen stepped down, Høyer's becoming the advisory editor of *Histochemical Journal* came as a surprise to no-one. Høyer retained his interest in stem cells, including gonadal stem cells, throughout his career (7-9). His main interest remained the fundamentals of histochemistry, including the importance of fixation and the use of a variety of blocking agents (10,11). Poul Erik was always willing to guide his fellow researchers, young and old, whenever they asked for help to resolve all kinds of histochemical issues. He was known to make incredibly meticulous reviews of their articles. Once an editor, always an editor.

Morten Møller also began his career at CHL in the early 1970s (see 3.2).

Bo van Deurs was recruited to the Anatomical Institute in the mid-1970s. He specialised in immunohistochemistry at the ultrastructural level and wrote a thesis in 1981 (12). His field of research focused on endo- and exocytosis of proteins. In a major, long-term international project, working in particular with Kirsten Sandvig from Oslo University and using a number of knock-out mouse models and *in vivo* manipulation of cultivated cells, Bo van Deurs made an important contribution to our understanding of the mechanisms involved in protein secretion.

Ole William Petersen was recruited to the department in 1983 (see below).

3.1.2: Kjeld Møllgård's research at CHL

In August 1968, **Helge Andersen** had finished a long thesis project and had been asked to describe the histochemical background for the development of the human adenohypophysis. He received this request from the editorial committee (Graumann, Lojda, Pearse, Schiebler) at a new and ambitious histochemistry journal, *Progress in Histochemistry and Cytochemistry, Gustav Fischer Verlag.*

Kjeld Møllgård (KM) had previously expressed an interest in working with human brain development. At this juncture, an introduction to histochemistry and human embryology based on pituitary development was therefore precisely what he needed (13-15). The future looked bright. Helge Andersen had innumerable histochemical methods running in the lab. He also had basic material available, e.g. hundreds of paraffin blocks from extremely well-fixed human foetuses from gynaecological units in the Copenhagen area. There was also a weekly influx of new foetal material that could be used for direct cryomicroscopy and electronmicroscopy, for which **F. v. Bülow** was responsible. Early in the process, KM discovered a particularly active and quite unknown area of the human foetal brain, the subcommissural organ, situated directly above the pituitary gland. With Helge's assistance, he used many of the laboratory's histochemical methods to characterise this organ in detail (16). This research included fluorescence microscopy of the organ's serotogenic innervation and electron microscopy, and later led to a thesis (17) that was defended at Lund University in Sweden in 1979.

In the following years, the department was responsible for broad-based courses in basic histochemistry. External members from other active research teams were invited as teachers. Thus young and active pathologists, **Per P. Clausen, Grete Krag Jacobsen** and **Marianne Jacobsen** took part in running the courses. They taught immunohistochemistry and started collaborating with members of CHL, a partnership which resulted in the earliest investigations of plasma proteins, including alpha foetoprotein in the human foetal brain (18). From this time, immunohistochemistry was brought into use in earnest at CHL. Marianne Jacobsen was a frequent visitor and there was a regular flow of articles (19,20) from the early 1980s, which led to Marianne Jacobsen's thesis, which was defended in summer 1986 (21). The publications included comprehensive counts of plasma protein-positive epithelial cells from human foetal choroid plexuses (Figure 3.1.2.1).

The histochemical work produced by CHL in the mid-1980s reflected developments in the research field and included not only classical histochemistry, but also fluorescence histochemistry, immunohistochemistry and quantitative histochemistry using "up-to-date" EM methods, including immunogold labelling. During this period, Helge Andersen's persistence and enthusiasm was indispensable. KM inherited Helge's collection of foetal material and expanded it over the years with ample help from colleagues. It grew to several thousand paraffin blocks and more than 100,000 paraffin sections. In recent years, KM has joined the confocal microscopic era and the brain sections from the many human foetuses are still in use on a daily basis (Figure 3.1.2.2) and the rate of publication is increasing (22,23).

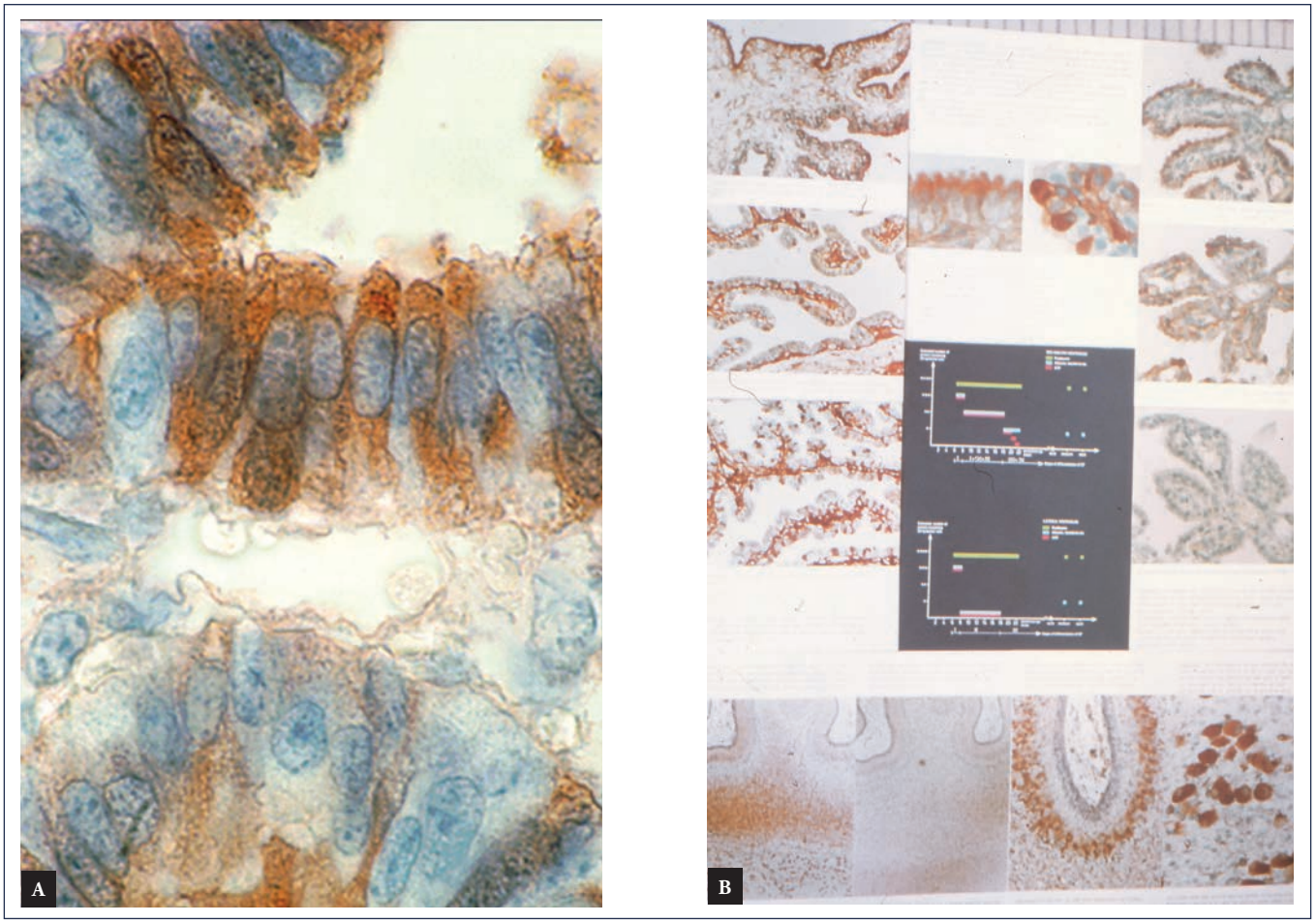


Figure 3.1.2.1. The illustration on the left shows immunoperoxidase staining of albumin-positive choroid plexus epithelium from a human embryo. Albumin is transported through a sub-population of epithelial cells from blood to the cerebrospinal fluid. To the right, a photo of a poster presentation of the results described in ref. 18.

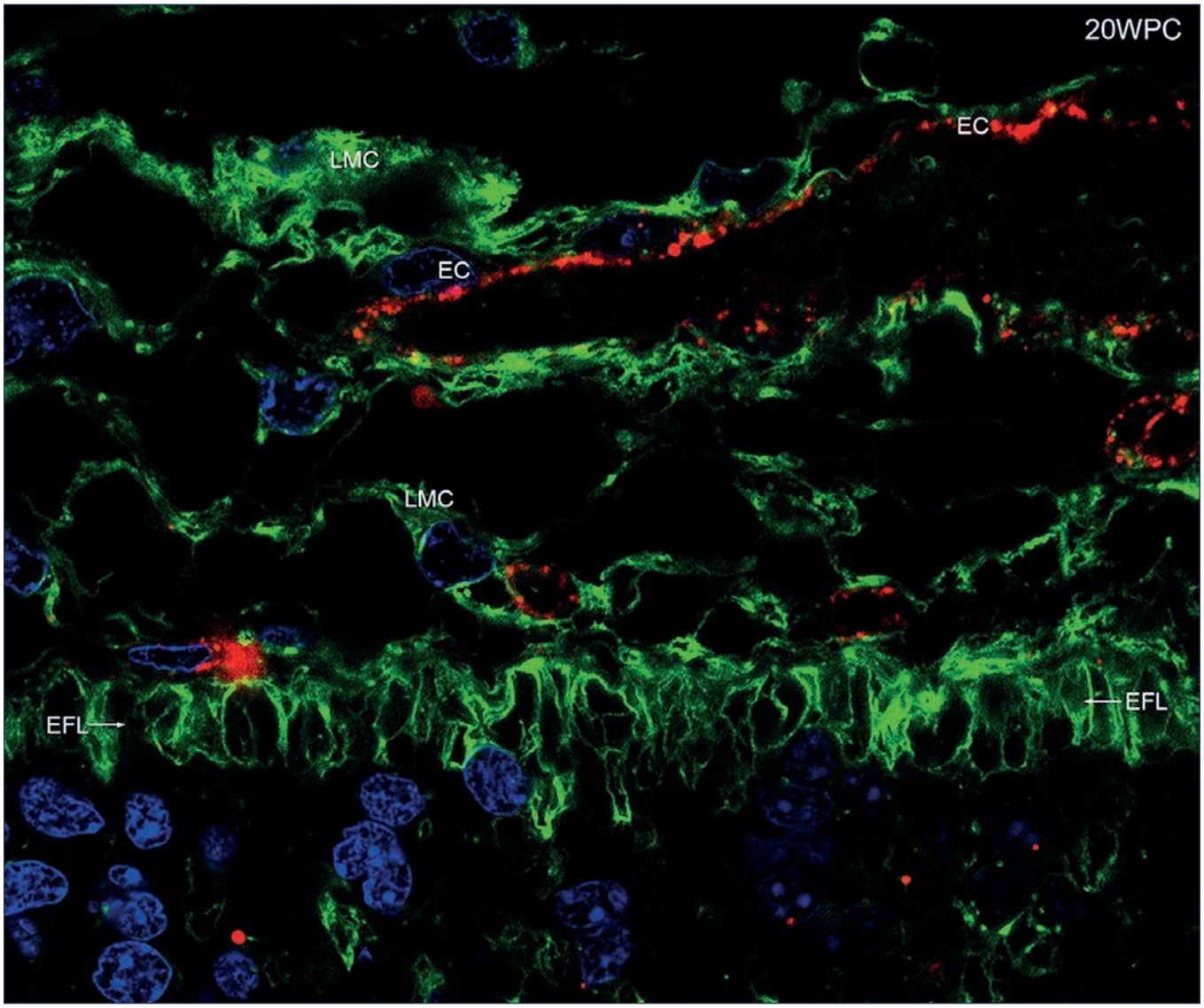


Figure 3.1.2.2. The outer surface of a human foetal brain and leptomeninges which signal via morphogens and a variety of hormones. The image, captured with confocal microscopy, shows a double immuno-labelled section from the cerebral cortex of a 21-week-old foetus. WPC stands for "weeks post conception". Stain for YKL-40 is green and for von Willebrand factor red. YKL-40 stains the membranes in the end feet layer (EFL) and leptomeningeal cells (LMC). Endothelial cells (EC) are stained red (22,23).

3.1.3: Ole William Petersen's research at CHL

While working at the Institute of Medical Anatomy, **Ole William Petersen** (OWP) also worked shifts at the Finseninstituttet on Strandboulevarden in Copenhagen. Here he learned about cancer and was introduced to another institution in the same building, Fibiger Instituttet. Fibiger Instituttet was the Danish Cancer Society's research centre in Copenhagen. Having read an article in the Danish medical journal, Ugeskrift for Læger, on the difficulties of cultivating and identifying cells from cancer patients, he approached the department of Tumor Endocrinology.

He speculated that some of the histochemical technology at CHL could be applied to such cultures. To a certain extent, the department of pathology had already adressed the issue at Rigshospitalet (RH). **Henning Jensen** had already written a thesis *(Enzymhistokemiske undersøgelser af fibroadenomatose og mammacarcinoma (Enzyme histochemical examinations of fibroadenomatosis and mamma carcinoma*) with a view to finding new markers for the early stages. Helge Andersen was, incidentally, one of the official opponents. Professor **Torben Schiødt** at RH was also interested in the early stages of cancer. OWP was introduced to both of them.

This was the advent of OWP's thesis project, in which he also had the pleasure of frequent contact with Senior Physician **Dorthe Francis** at Bispebjerg Hospital and Senior Physician **Klaus Hou-Jensen** at Finseninstituttet. OWP established primary cultures from breast cancer tissue biopsies and, working with **Bo van Deurs**, characterised the cells by comparing them with stained tissue of origin. His methods were based on the well-characterised enzyme histochemical methods he had learned at CHL. He developed a special interest in oxidoreductases and, in particular, glucose-6-phosphate dehydrogenase and how it is affected by oxygen. OWP was introduced to the stringency of Poul Erik Høyer, for whom accuracy and attention to detail were absolutely indispensable. OWP's thesis was published in 1990 (24).

3.1.4: From CHL to ICMM

The scientific methods and approaches developed by Helge Andersen at CHL in the early 1960s have been passed on to later generations of scientists. They form the basis for a great deal of research science and many theses, in particular, in the field of breast cancer research.

In 2007, parts of three departments, the previous institutes of medical biology, medical physiology and medical anatomy, were merged to form *Institut for Cellulær og Molekylær Medicin (ICMM) (The Department of Cellular and Molecular Medicine*, which included the following research fields: morphogenesis and differentiation, medical genetics, RNA and genetic medicine, glycomics and molecular ageing.

Ole William Petersen is currently leader of ICMM.

Most of the methods used now are new but the creativity of Helge Andersen's era lives on.

For further information, visit http://icmm.ku.dk/.

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3.2: THE USE OF HISTOCHEMICAL METHODS IN INVESTIGATING THE CENTRAL NERVOUS SYSTEM

by Morten Møller

3.2.1: The classic silver staining techniques

The classic silver impregnation method for staining nerve cells was developed in the second half of the nineteenth century by the Italian physician, **Camillo Golgi** (1843–1926). In 1873, he impregnated a thick section of nerve tissue with potassium dichromate and silver nitrate (1) and achieved microcrystallization of silver chromate on the nerve cell bodies and their dendrites (see Figure 3.2.1.1).

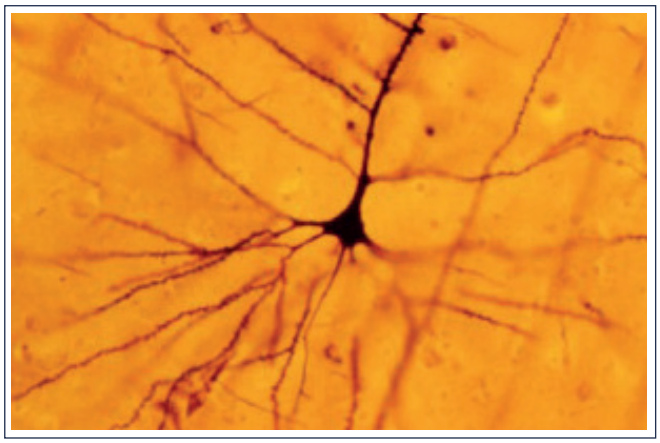

Figure 3.2.1.1. Pyramidal neuron from human cerebral cortex stained using Golgi's silver impregnation technique.

This reaction creates a very clear visualisation of the the neuronal cell body and its processes. Unfortunately however, this method only impregnates a small percentage of the neurons.

The Spanish neuroanatomist, **Santiago Ramón y Cajal** (1852-1934) (Figure 3.2.1.2A), used the Golgi staining method and succeeded in mapping most of the nervous system using this method. Santiago Ramón y Cajal is often – and deservedly – described as the father of modern neuroscience.

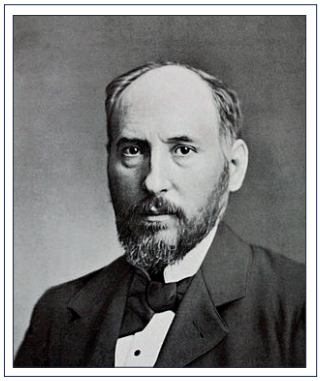

Figure 3.2.1.2A. Santiago Rámon y Cajal

Cajal improved the Golgi staining method by adding osmium tetroxide to the potassium dichromate solution (the rapid Golgi method). Later, Cajal developed a neuron staining method of his own (2), in which the neuron is treated with silver nitrate and then developed photographically. Finally, he developed a method for staining astrocytes, in which he impregnated the tissue with a solution of gold chloride and mercuric chloride (Figure 3.2.1.2B).

Cajal shared the 1906 Nobel Prize with Camillo Golgi. Around 1920, one of Cajal's students, **Pío del Río Hortega** (1882–1945), developed a silver impregnation method for the nervous system's third glial cell (see Figure 3.2.1.3), the microglial cell (3).

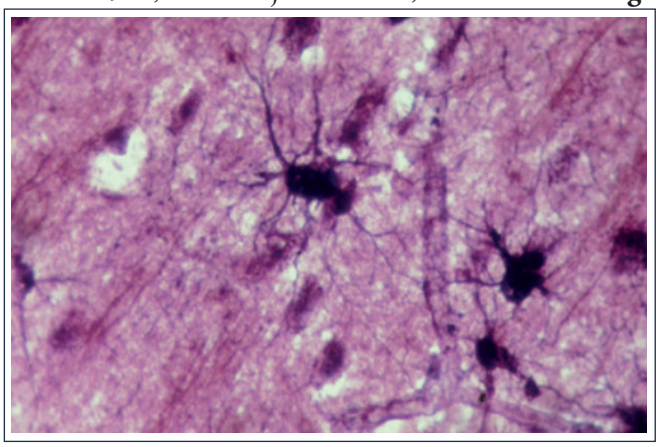

Figure 3.2.1.2B. Cajal's gold-sublimate method for astrocytes. Note the end feet from the astrocytes covering the capillary walls.

Subsequent modifications of the silver impregnation technique have resulted in stainings, which are specific for degenerating myelin (Marchi) or degenerating axons (Nauta). In the last century, the Nauta method has been used in experiments to demonstrate connections in the brain and spinal cord.

As many brain diseases cause degeneration in the central nervous system, which produces a variety of precipitations in the cerebral parenchyma, neuropathologists have since developed modified impregnation techniques to reveal the presence of pathological deposits in the brain, e.g. Bielschowsky's method to demonstrate neuritic plaque (Alzheimer's and Pick's Disease) and Gallya's staining for neuronal plaques and neurofibrillary tangles.

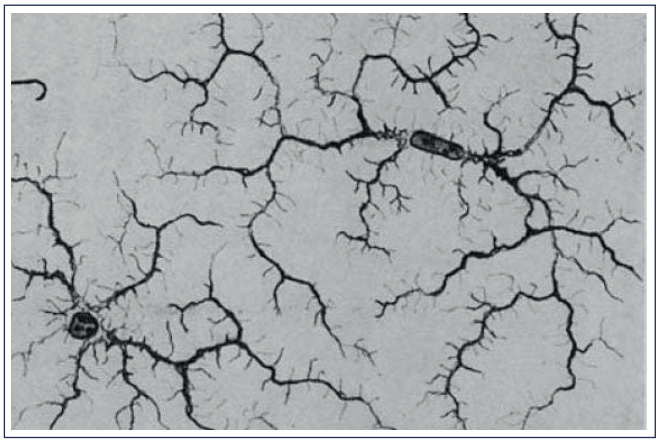

Figure 3.2.1.3. Microglial cell stained using Río Hortega's silver carbonate method.

At the end of the 20th century, based on the classical metal impregnation techniques for neurons, Professor **Gorm Danscher** and his coworkers developed a number of new techniques (autometallography) to detect different metal ions in the brain cells. See the very specific localisation of zinc in figure 3.2.1.4.

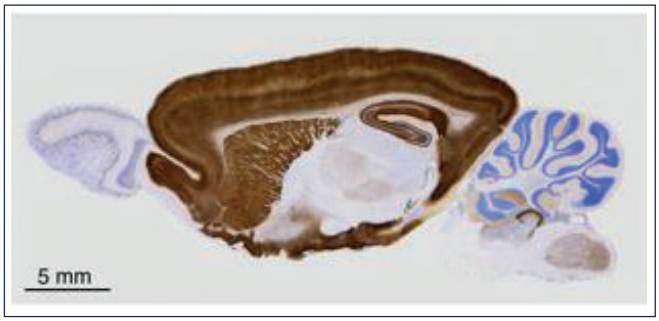

Figure 3.2.1.4. Sagittal plane section through a rat brain, in which neurons containing Zn (a brown reaction product) are visualised using autometallography with the section counter stained with toluidine blue.
(Danscher F, Stoltenberg M. Progr Histochem Cytochem 2006;41:57-139).

3.2.2: The Falck-Hillarp fluorescence method

In the early 1960s, before highly specific antibodies were developed for immunohistochemistry and when sensitivity of the methods was low, two Swedish research scientists, **Bengt Falck** and **Nils-Åke Hillarp**, working at the histological institute in Lund, developed a histofluorescence technique for visualising monoamines (dopamine (Figure 3.2.2.1 and 3.2.2.2), noradrenalin and serotonin).

The original Falck-Hillarp method relied on exposing freeze-dried tissue to formaldehyde vapour so that the

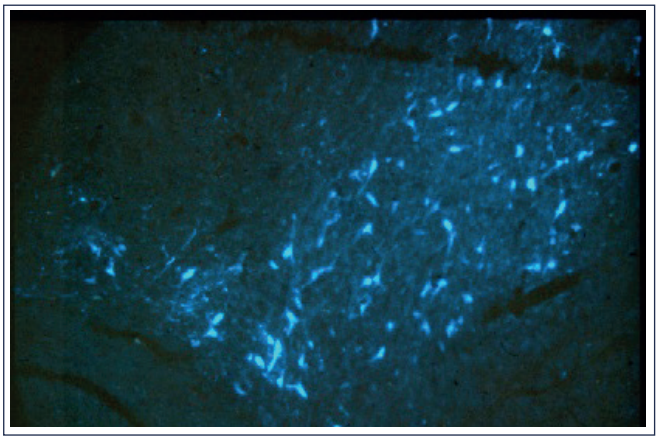

Figure 3.2.2.1. Dopaminergic neurons in substantia nigra. Falck-Hillarp method. (Møller, unpublished)

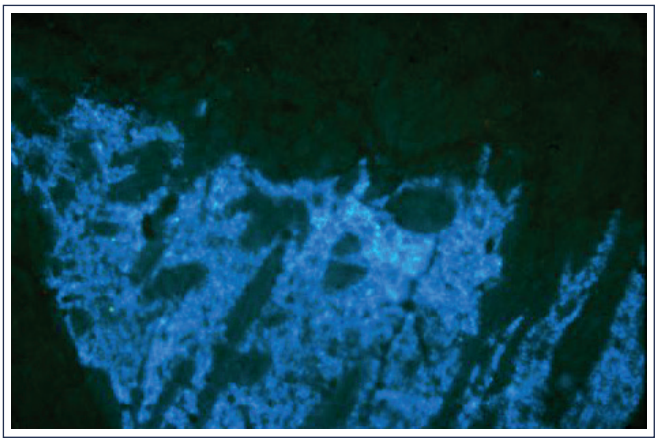

Figure 3.2.2.2. Dopaminergic innervation of striatum. Falck-Hillarp method. (Møller, unpublished)

dopamine, noradrenalin and serotonin were converted into iso-quinolin molecules, which emit a yellow-green and clear yellow fluorescence, respectively, under the microscope. Early in our scientific career, Kjeld Møllgård and I travelled to Lund to learn this technique, which we then set up at The Institute of Medical Anatomy for our investigations of the innervation of the pineal gland. The problem with the Falck-Hillarp technique is that the background is dark and that it is therefore difficult to identify non-fluorescent structures in the section.

Even though there were many attempts to develop new fluorescence techniques for other transmitters than the indol- and catecholamines, none was successful. Instead, immunohistochemistry developed, due especially to the production of antibodies with growing specificity and avidity. Thus, **Finn Geneser, Jens C. Sørensen and Carsten Reidies Bjarkam**, all from Aarhus University,

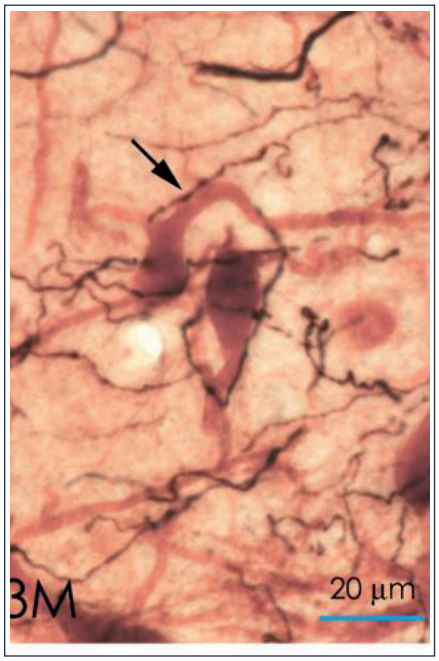

Figure 3.2.2.3. Image of rabbit hippocampus showing two calbindin-positive neurons (brown) in contact with dark varicose serotonergic fibres. (from Bjarkam CR, Sørensen JC & Geneser FA. Hippocampus 2003;13(1):21-37).

have used antibodies against serotonin and have mapped the serotonin innervation of hippocampus in the rabbit (Figure 3.2.2.3).

3.2.3: Own investigations of the cerebral photo-neuroendocrine system using histochemical methods

Background for the laboratory's research

The cerebral photo-neuroendocrine system comprises:

a) an oscillator in the midbrain, which imposes a 24-hour rhythm on other areas of the central nervous system,

b) the eye, through which light is transmitted to the oscillator. Light from the eye may stimulate the phase change in the oscillator itself.

The oscillator is located in a collection of nerve cell bodies (a nucleus) in the midbrain (diencephalon), which is called the suprachiasmatic nucleus (SCN) (Figures 3.2.3.1 and 3.2.3.3) because it lies just above the optic chiasma.

The most important projection from the SCN goes to the paraventricular nucleus in that part of the brain we call hypothalamus. Nerve fibres run from here and all the way down through the brainstem to a nucleus in the lateral horn of the spinal thoracic segments and further out to the truncus sympaticus, where the next synapse is found in the superior cervical ganglion (Figure 3.2.3.1).

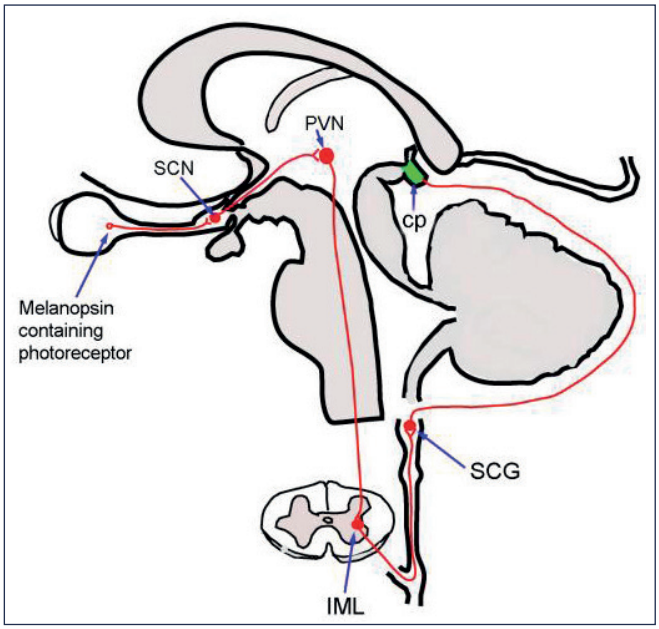

Figure 3.2.3.1. Drawing of the anatomical pathways from retina to corpus pineale Via the retinohypothalamic pathways in nervus opticus, retinal photoreceptors containing melanopsin send information about ambient light conditions to the suprachiasmatic neurons (SCN) in hypothalamus. From SCN, the impulses are sent on via the paraventricular nucleus (PVN) in hypothalamus to the autonomous nerve cells (intermediolateral nucleus (IML)) located on the lateral horn of the spinal cord's uppermost thoracic segments. From here, preganglionic nerve fibres are sent to ganglion cervicale superius (SCG) in the sympathetic chain, from which postganglionic sympathetic neurons run to corpus pineale (cp). The noradrenalin signal molecule binds to β1 receptors on melatonin-producing pinealocytes, stimulating the release of melatonin.

From here, the sympathetic nerve fibres run with arteria carotis interna into the cranium and stimulate the pineal gland to release the hormone, melatonin (4).

In the retina, there are in addition to rods and cones, specific photoreceptors which contain a photopigment, melanopsin. These receptors, which were discovered only within the last five years, send nerve fibres to the SCN and can (as described above) phase shift the activity of the nerve cells in this nucleus.

Own investigations

In our investigations of the photo-neuroendocrine system, we used the Falck-Hillarp fluorescence technique, immunohistochemical methods and *in situ* hybridization to:

1. map the neuro-anatomical connections from the eye to the suprachiasmatic nucleus (SCN) and further from the SCN to the paraventricular nucleus in the hypothalamus, from this nucleus to the lateral horn of the spinal cord and from here to ganglion cervicale superius, from which sympathetic strands reach the pineal gland (Figure 3.2.3.1 and 3.2.3.5) (4-7).

2. detect neurotransmitters and receptors in SCN and the pineal gland (Figures 3.2.3.2, 3.2.3.3 and 3.2.3.4) (8-12).

3. detect the Circadian rhythm in the expression of genes in SCN, pineal gland, cerebral cortex and cortex cerebelli.

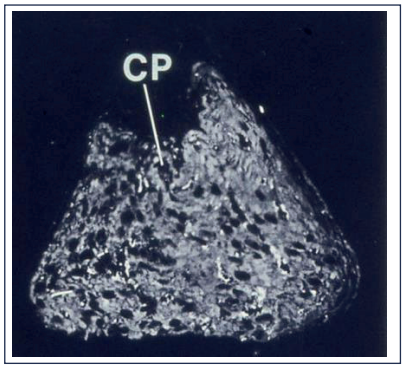

Figure 3.2.3.2. Histofluorescence image of the pineal gland in a gerbil. The strongly fluorescent nerve fibres, which contain noradrenalin, are seen as beaded strings between the less fluorescent pinealocytes.

Visualisation of the suprachiasmatic nucleus via immunohistochemical demonstration of neurons containing neuropeptides, e.g. vasopressin (Figure 3.2.3.3), vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP) and pituitary adenylate cyclase-activating peptide (PACAP) (Figure 3.2.3.4). All of these peptides influence the function of SCN. It is now clear that PACAP is the neurotransmitter in the route from the eye to SCN (Figure 3.2.3.4) (12).

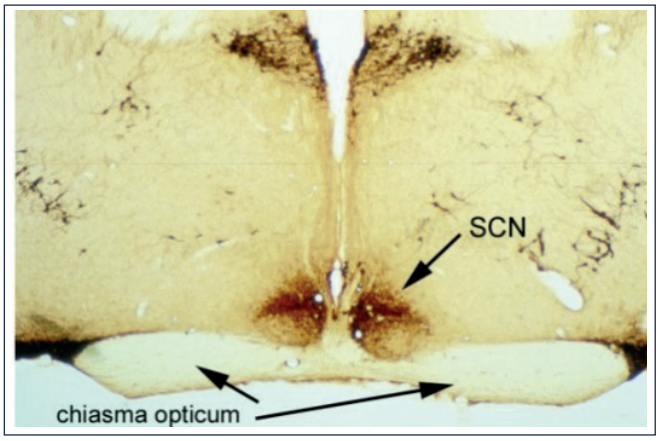

Figure 3.2.3.3. Coronal section through the forebrain of the rat at the level of the optic chiasma. The suprachiasmatic nucleus (SCN) lies above the optic chiasma and is visualised immunohistochemically using a vasopressin antibody.

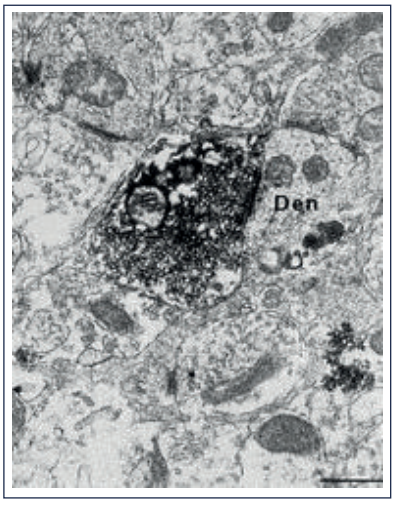

Figure 3.2.3.4. An immunocytochemical reaction at electron microscopic level. The image shows a nerve terminal in SCN, which is positive (Black colour in and around the small neurotransmitter vesicles) for the neuropeptide, PACAP. Den = dendrite.

Mapping the connection from the eye to the suprachiasmatic nucleus by means of neuronal in vivo injection of a tracer, cholera toxin (Figure 3.2.3.5). The tracer is taken up by cells in the retina and transported anterogradely to the suprachiasmatic nucleus. The tracer is detected by using immunohistochemistry and an antibody against cholera toxin.

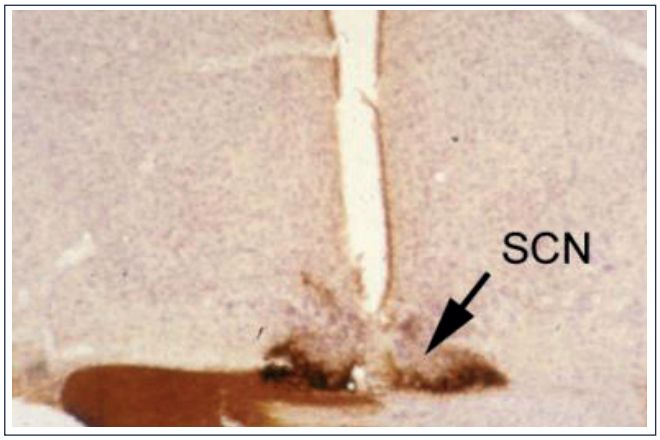

Figure 3.2.3.5. A tracer, choleratoxin, is located in the optic chiasma and the ventral part of the suprachiasmatic nucleus (SCN) after injection in the right eye of a gerbil. Coronal section through hypothalamus.

In the pineal gland, we have demonstrated diurnal variation in gene expression of aralkylamine N-acetyltransferase, which is the rate-limiting enzyme in melatonin synthesis. Our experiments show that a higher secretion of melatonin at night is due to restoration of the enzyme. If the sympathetic input to the pineal gland is removed, the increase in mRNA expression – and therefore that of the enzyme itself – disappears.

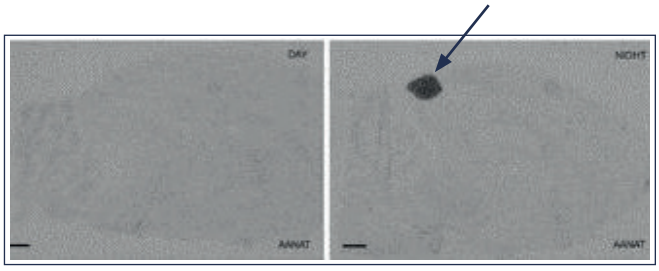

Figure 3.2.3.6. Medial section through rat brain, which has been hybridised with a radioactive cDNA-probe, which binds to mRNA encoding for aralkylamine N-acetyltransferase, the rate-limiting enzyme in melatonin synthesis. The image on the left is from a rat killed in the light at midday, while the image on the right is from a rat killed in the dark just after midnight. The arrow points to the pineal gland at high density, i.e, expressing the presence of more mRNA at night. Bar = 1 mm.

Demonstration of diurnal variation in gene expression in SCN, pineal gland, cerebral cortex and cortex cerebelli.

The arrival of the *in situ* hybridization technique (Figure 3.2.3.6) was a revolutionary step in the laboratory's research. For the first time, we had a technique, which could quantify a biochemical parameter in a cryostat and paraffin-embedded section. The process requires the use of a radioactive probe, in which the radioactive signal can be quantified on a film, which is exposed by the radioactive radiation. Furthermore, it is possible to control the signal by measuring the quantity of mRNA using a method called qRT-PCR (quantitative reverse transcription polymerase chain reaction) and the quantity of protein can be measured using western blotting.

By exposing laboratory mice to light, we were able to show that light stimulation changes the gene expression that is involved in regulating the function of the clock in the SCN (13, 14).

We have also demonstrated the presence of several transcription factors in the pineal gland, which modulate production of melatonin (15-17).

Finally, we have shown that there are "peripheral clocks" in neocortex and cerebellar cortex (18), whose activity is controlled by the central "clock" in SCN, but which have a rhythm of their own if SCN is damaged. The peripheral "clocks" in neocortex may be involved in mental disorders as disrupted circadian rhythms are symptomatic of several of these disorders.

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3.3: IMMUNOFLUORESCENCE STAINING IN DENMARK
by Jørgen Rygaard and Erik Dabelsteen

The work of writing this article is split between two authors: **Erik Dabelsteen** (ED) and **Jørgen Rygaard** (JR). Section 3.3.1 describes the rise of interference filters. It is purely technical and was written by JR. The refinements regarding the use of this method in specific fields of research and diagnostics (Sections 3.3.2-3.3.3) were written by ED. Section 3.3.4 is written by JR. To tell the truth, JR has always been more interested in a nude mouse without thymus.

3.3.1: The first time I ever saw a fluorescence microscope was in February 1964...

I graduated in June 1962 and began my career with an internship at Frederiksværk Hospital, a small hospital with only four doctors: a senior physician, a specialist registrar, a first-year intern and me. There was a day shift and a night shift. During the summer the number of patients doubled due to hordes of holidaymakers. The hospital could only afford to staff the laboratory and X-ray department during the day so I gained appreciable practical experience of – and developed a liking for – laboratory work. After a couple of months' military service as a sergeant in spe, I applied for a job at the bacteriological department at Statens Serum Institut (SSI). The job went to someone else but I received a very friendly letter telling me that a job in the treponematosis unit would be coming up in three months' time. I politely accepted the offer and then spent the interim three months working as a freelance TV producer (three programmes on psychological disorders!).

From 1 February 1964, in the wake of this particularly hectic period (first working at the hospital, military service and then as a TV producer), the peace and quiet of working on Amager Boulevard came almost as a shock. The department's senior expert delivered a whole pile of books: Everything I needed to know about treponema pallidum. Take your time. Read these.

By the summer, I was desperate. I missed activity. Saw a job ad in Ugeskrift for Læger for a position as municipal physician on a very small island called Fur. I put the wife and kids into the car and drove north...

My decision came like a flash of lightning. The man on the ferry selling tickets asked: "Single or return?" I was in no doubt: Return!

And I was back at the treponematosis unit. The unit took care of all syphilis diagnostics in Denmark and the North Atlantic territories. It had records of all positive Wasserman (WR) reaction tests since the beginning of time. It was also an international WHO reference centre. No citizen, regardless of their age, was ever admitted to a Danish Hospital without a WR and a haemoglobin test was performed. The basis of the serological testing was the Wasserman test, which was a complement fixation reaction, using an antigen, Cardiolipin, extracted from bovine heart. The test was also positive in a number of autoimmune diseases. The specificity was determined by two tests: TPI (Treponema pallidum Immobilisation test) and TPA (Treponema pallidum (fluorescent) Antibody test). It was in connection with the second of these tests that the unit's one and only fluorescence microscope enters the story. This was a not-very-refined Leitz microscope with a 100 W high-pressure mercury lamp (HBO100W).

The bacteria used in both tests were cultivated by injecting bacteria into the testes of rabbits. I felt sorry for the poor rabbits and worked hard to cultivate treponema bacteria in diffusion chambers and in other ways. Without success. I find some consolation in the knowledge that today – more than 50 years later – this issue has not yet been resolved.

My next project involved examining how treponema spread in rabbits. The unit had many, very positive samples of patient serum. It was a piece of cake: take an imprint or cryosection as an antigen, patient serum in the first step and then FITC-conjugated anti-human rabbit IgG, produced by repeated immunizations of rabbits. (These conjugates could become astonishingly potent. We could dilute them as much as 800 times). To no avail. In my laboratory, I had a large, brand new Zeiss research microscope, which also had a HBO 100 W light source and a BG5 glass filter as the primary filtering element.

The result is perfectly described in two lines of *The Dying Child*, a poem by Hans Christian Andersen: "Green, gold, and red, are floating all around me; They are the flowers the angel scattereth." Not exactly flowers scattered by an angel. I was plagued by autofluorescence, which resulted from using old-fashioned glass filters with a very broad transmission field. The BG5 filter transmitted everything in its path, from about 350 nm to just over 500 nm.

A few years earlier, the unit had hosted a WHO training course in immunofluorescence techniques. It was taught by the Scottish-Australian Professor **R.C. Nairn** from Monash University in Melbourne. Nairn had written the Bible of fluorescence technique, *Fluorescent Protein Tracing*, a copy of which I received in connection with the training course. I was astonished when I realised the divergence between the BG5 filter transmission curve and the FITC absorption curve with absorption maximum at 488 nm. Nowadays everyone knows this... (Figure 3.3.1.1).

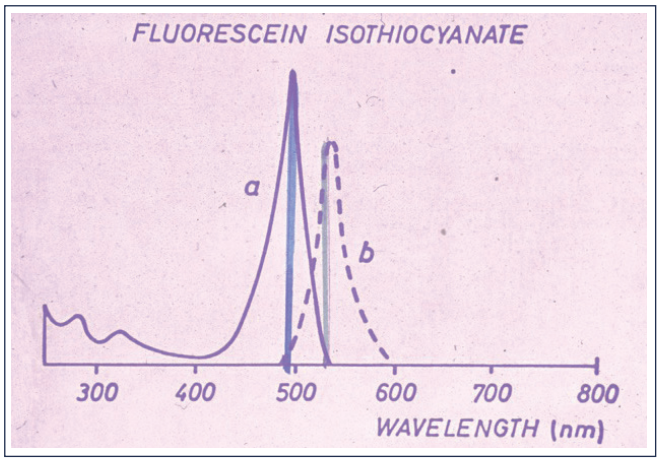

Figure 3.3.1.1. Absorption (a) and emission curves (b) for FITC. Illustration from Rygaard & Olsen's first publication (1).

The filter catalogues had nothing better to offer than BG5. ATV, The Danish Academy of Technical Sciences, ran a number of specialist units, including "Belysningsteknisk Institut" (The Department of Lighting Technology). I called them. Did they have light source with maximum at 488 nm? They were aware of the issue, but no. Call Optisk Laboratorium (the optical laboratory).

Which I did. The boss at the optical laboratory, civil engineer **Werner Olsen** (WO) (1927-2015) confirmed that they did have a narrow band filter with a transmission maximum of 488 nm.

For that matter, they had filters in many different wave lengths (Figure 3.3.1.2).

"What are they used for?," I asked, amazed.
"Nothing at all, they are just lying in a drawer!"
The next day, WO came over to SSI with his filter. And I saw the light!

Figure 3.3.1.2. A selection from Optisk Laboratorium's collection of filters.

In addition to WO, Optisk Laboratorium at Denmark's Technical University (DTU) also employed a Norwegian engineer with Asperger's Syndrome, **Rofsdahl**. He had acquired the most advanced Texas Instruments calculator available. It had certainly cost an astronomical sum (and undoubtedly had less capacity than my youngest grandchild's iPad).

The filters Rofsdahl calculated were created by coating in vacuum with alternating high-and lowrefractive salts. The process was controlled using a reference beam calculated by Rofsdahl. A civil engineer, **Poul Smith**, took care of the practicalities and in addition there was a secretary. When, thanks to the filters, the sections of tissue were black as coal, I asked for a filter with light enough for orientation.This turned out to be a filter with a narrow chink at about 630 nm, which gave the same effect as was later obtained with Evan's Blue in the final rinse.

"Can I get filters for Rhodamine?" "Sure. You just have to ask."

Now, when I look around me in the world of instruments just less than 50 years later, I see interference filters everywhere. Sometimes, just for a moment, I regret that we did not take out the patent on our filters. Today, it would be a natural requirement of the granting authorities. Things were done differently back then. WO opposed the idea of applying for the patent: "It'll cost a whole lot of time and a pile of money. And people who wish to infringe our patent rights, will do it anyway!"

He was probably right. Instead, we wrote a short article in *Acta Pathologica Microbiologica Immunologica Scandinavica: Interference filters for improved immunofluorescence microscopy* (1-3).

The cat was now out of the bag and we had to find potential buyers and users. I called Zeiss' Danish department in Copenhagen. A highly competent salesman, Mr. **Heger**, announced his arrival at SSI. I rigged up a microscope with a standard 60 W bulb, a 488 nm filter with a bit of red and a yellow glass filter as the secondary filter. I then borrowed some sections from **Allan Wiik** at the Autoimmunology Laboratory at SSI, which had been used to test for ANF, anti-nuclear factor.

The microscope was switched on. Mr Heger entered, took a rapid glance in the microscope, turned on his heels and, as he left the building, he said, "It's brilliant!" A few days later, he announced his arrival again, this time accompanied by Dr. **Kraft**, Director of Development at Zeiss in Göttingen.

Before their visit was over, they and WO had agreed to send a large number of filters to Göttingen.

The second large microscope manufacturer, Leitz, reacted totally differently. In the next two editions of their newsletter, *Leitz Mitteilungen,* Leitz lashed out and delivered a stream of arguments against using interference filters of any kind. These arguments flew in the face of the laws of physics. I suppose they had a huge stock of HBO 100 high-pressure cannons to dispose of. Then, after two editions of hopeless arguments, Leitz gave in to the laws of physics and interference.

WO and I signed up to attend the spring meeting of The Royal Microscopical Society in London. Dr. **A.H. Tomlinson** from Radcliffe Infirmary in Oxford showed polite interest. He himself had played around with some inferior metal interference filters. The other attendees did not deign even to glance at the two fools from *the Continent*.

Undaunted, in May 1970, we travelled to a meeting in Stockholm, arranged by The Swedish Society for Microbiology and The New York Academy of Sciences. The meeting was held at the Wenner-Gren Centre. We took a rather provocative step and set up our microscope with a 60 W bulb and interference filter with one of Allan Wiik's ANF sections on the ground floor just inside a 4 or 5-metre high gable end in glass. People seemed to think we were crazy and were reluctant to take a look.

Later, attendees were invited to dinner at the homes of our Swedish colleagues. This was a generous social gesture. WO and I ended up at the beautiful home of **Eva** and **Georg Klein** on an island, Lidingö. We told our hosts of the blessings afforded by interference filters and were invited to visit the tumour biology unit at Karolinska. Enthusiastic young scientists sat in small black boxes looking at surface antigens in Burkitt's lymphoma. They tried out the interference filters and dropped them in disdain: The detection limit moved right out of their arbitrary scale. This meant that they would have to start from scratch!

In July 1967, I started work as the youngest registrar at the pathological anatomical department at Københavns Kommunehospital (KH). I worked there and part-time at SSI until 1969. To continue the story, I was one day sitting in my physician's office at KH. The door opened and without further ado, **Per Christoffersen, MD** said: "Rygaard, walk over to the School of Dentistry and have a chat with a dentist called Dabelsteen." He closed the door. Understood? Loud and clear.

I trotted off to the School of Dentistry in Universitetsparken.

Erik Dabelsteen and I have now worked together for more than 45 years.

3.3.2: ABO blood group antigens in normal and malignant oral mucosa

A number of changes occur in the oral mucosa, which are potentially malignant. Research has shown that only 10% of these changes actually develop into carcinoma, which means that we need to be able to identify precisely which of these changes subsequently turns malignant (4).

During the latter half of the 1960s, **J.J. Pindborg**, a professor at the School of Dentistry, received an NIH research grant to study potentially malignant conditions in the oral mucosa. One of the projects aimed to find histological markers for early malignant development.

As early as in 1926, **Karl Landsteiner** demonstrated that normal epithelial cells contain ABO blood group antigens (5). In 1930, using tissue extracts and immunological methods, **Olaf Thomsen** at Rigshospitalet (RH) discovered that these antigens disappeared in connection with the development of carcinoma (6). In 1969, this particular finding led to new research, using an agglutination test on histological sections, which showed that not only blood type A and B antigens, but also their histological precursors disappear in cancer of the cervix (7). This research generated a desire to investigate how changes in the presence of A and B blood type antigens could be used as markers for incipient malignant development in pre-malignant changes in oral mucosa.

The study began by using a conventional two-step staining method with blood type antibodies from the blood bank at SSI and with FITC-labelled antibodies purchased from a commercial source. As usual in the mid-1960s, the fluorescence microscope was equipped with a glass primary filter and a conventional dark field condenser. Formalin-fixed and paraffin-embedded tissue could be used because ABO antigens are carbohydrate structures that are not affected by routine histological preparation techniques. There were, however, a series of challenges. The best antibodies available from the blood bank were of the type lgM because they were selected on the basis of their capacity to agglutinate erythrocytes. The secondary antibodies, which had to be used in the immunofluorescence technique, were mainly of type lgG and conjugation was poor. Fluorescence intensity was also poor because the primary filters were not appropriate to use with FITC and the dark field condensor obscured most of the light emitted from the light source.
Once contact was established to **Jørgen Rygaard** and **Werner Olsen**, who had developed new primary fluorescence filters (1), **Agnete Ingild** and **Niels Harboe** from Proteinlaboratoriet, which had high titer and well-conjugated secondary antibodies, and registrar **Henning Sørensen** from Retsmedicinsk Institut (Department of Forensic Medicine at the University of Copenhagen), the team succeeded in demonstrating that blood type antigens A and B are normally found in oral mucosa as a membrane antigen in the squamous epithelium (Figure 3.3.2.1), and that their presence in the epithelium is regulated by secretor genes, which also regulate the presence of blood type antigens in saliva (8,9). Using ferritin-labelled antibodies and electronmicroscopy, cell membrane localisation was confirmed (10). The team also succeeded in demonstrating that most carcinomas lose their ABO antigen and that in many potentially malignant diseases the antigen is also lost (Figure 3.3.2.2.) (11).

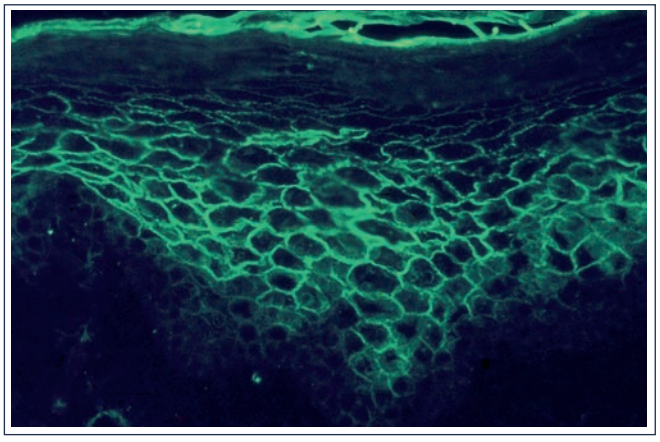

Figure 3.3.2.1. Blood type A antigen in normal lip mucous membrane.

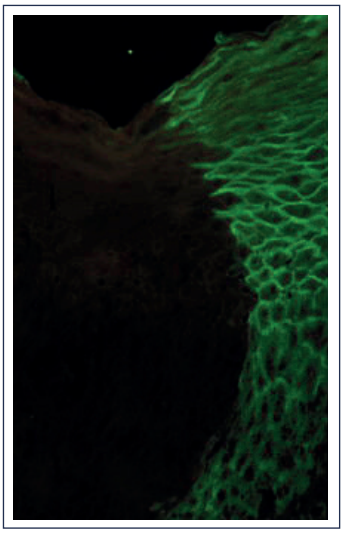

Figure 3.3.2.2. Staining of blood type A antigen in the transitional phase between normal and malignant oral mucosa, where the reaction is negative.

The ABO antigen determinant is the terminal section of a carbohydrate chain that is built in steps adding individual carbohydrate structures, a process that is catalysed by more than 200 known glycotransferases.
In Seattle, **Sen-itiroh Hakomori** was studying changes in the cell membrane glycolipids in connection with malignancy. He succeeded in isolating the various stages of blood type antigen synthesis. In 1982, he produced monoclonal antibodies corresponding to each individual step in this synthesis (12). A partnership between Københavns Tandlægeskole (Copenhagen School of Dentistry) and Sen-itiroh Hakomori succeeded in tracking blood type antigen synthesis in multiple layers of the squamous epithelium. The team demonstrated that precursors of blood type antigens were present in the basal and parabasal cell layer (Figure 3.3.2.3), whereas the complete antigen was expressed in more differentiated cells. The changes found in malignant tissue corresponded

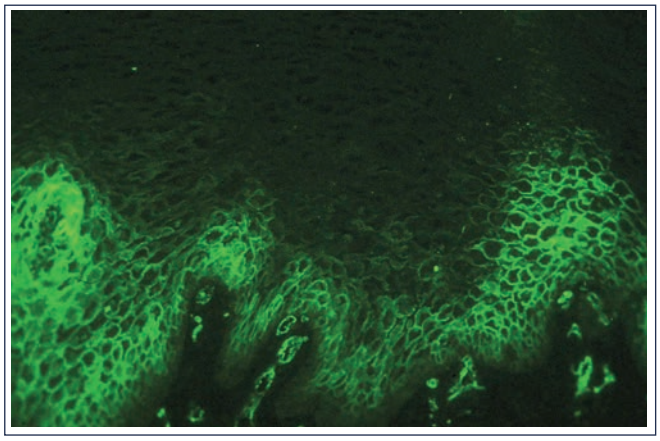

Figure 3.3.2.3. Staining of cheek mucosa for precursor to blood type A or B: H antigen (in O subjects). The staining is localised in parabasal cells in the epithelium.

to an interruption of the synthesis and an accumulation of blood type antigen precursors (12).

There is therefore a fine regulation of the blood type antigen synthesis, which follows epithelial differentiation. The investigators were **Poul Vedtofte** and **Erik Dabelsteen** (in Copenhagen) with **Sen-itiroh Hakomori** (in Seattle) and **Henrik Clausen** and **Ulla Mandel**, who were guest researchers from Copenhagen.

Working with Hakomori, Henrik Clausen cloned the ABO gene, which is a gene coded for a glycosyltransferase, which ads the terminal sugar onto the carbohydrate chain (13). Using A-gene transferase antibodies and immunofluorescence staining, the scientists could follow the synthesis of the carbohydrate structures on the epithelial cell surface, not only the antigen itself, but also the enzyme responsible for synthesis of the antigen (14). By combining immunohistochemistry with laser capture microscopy, in which selected areas of the histological section are removed for molecular biological analysis, it was shown that many but not all cases, in which there was a loss of A or B blood type antigen in oral cavity cancers, could be explained either by a loss of heterozygosity (LOH) in chromosome 9q34, which is locus for the ABO gene, or by hypermethylation of the ABO gene promoter (15).

The study of the blood type antigens in the tissue using immunohistochemical techniques was very soon extended to include a number of other tissues (16), which meant that there were comprehensive studies of bladder carcinomas, the endometrium, saliva glands, ventricle, colon, larynx, prostate and skin (17-22). Most of these studies did not only include blood type antigens AB and their enzymes but also analysed for the Lewis antigens, which are related to the ABO system (23).

3.3.3: Immunofluorescence in diagnosing bullous disorders of the skin and mucosa

In the mid-1960s, **Beutner** demonstrated that patients with two bullous disorders, pemphigus and pemphigoid, have autoantibodies in the blood, which react to the cell membranes and basement membrane, respectively, of the squamous epithelium (Figure 3.3.3.1). Using immunofluorescence techniques, it was shown that the patient's clinical symptoms largely correlated with the antibody titer in patient serum. However, in a bullous condition known as benign mucous membrane pemphigoid, which primarily affects the eyes and mouth, it was not possible to find autoantibodies in the same way as in pemphigoid. Using Jørgen Rygaard and Werner Olsen's fluorescence microscope supplemented with a Japanese Tiyoda dark field condenser (with moulded plastic toric lenses), Dabelsteen and Rygaard succeeded in demonstrating the antibodies present in these patients (24).

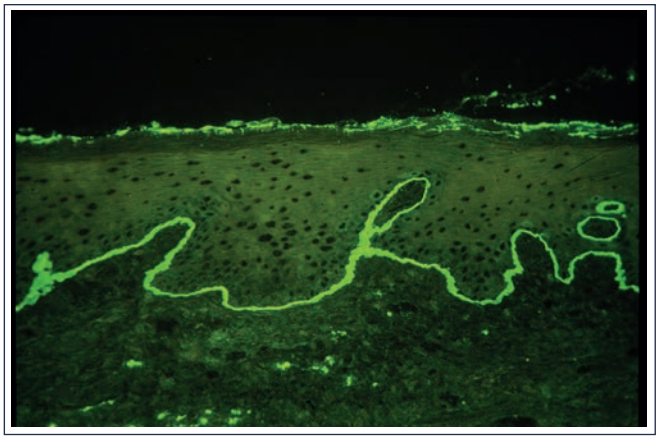

Figure 3.3.3.1. Bullous pemphigoid. Patient antibodies bound to guinea pig lower lip. Two-step staining method.

The antibodies were found in low titers, which probably explains why they were not demonstrated in conventional immunofluorescence systems. It later appeared that, in some cases, patients with benign mucous membrane pemphigoid have laminin-5 antibodies (laminin 332), while others have antibodies against other basement membrane proteins. It appears that the course of the disease in patients with laminin-5 antibodies creates more severe scarring. These findings are applied in diagnostics today by using a skin section where the epithelium is separated from the connective tissue by, e.g. EDTA as antigen. The laminin-5 antigen is located on the epithelial side, which can be seen in a fluorescence staining using patient serum.

3.3.4: The use of immunofluorescence in nephropathology

Until the middle of the 1900s, most of what we knew about morphological changes in kidney diseases was based on autopsy material and examinations of surgical specimens.

The introduction of percutaneous needle biopsy, performed in 1949 by Senior Physician, later Professor, **Poul Iversen** (1889-1966) and **Claus Brun** (1914-2014), then a registrar at Københavns Kommunehospital (KH) unit 3 (25), made it possible to obtain fresh material from patients who had clinical symptoms of kidney disease. The biopsy made it possible to correlate the patient's symptoms and clinical data with light microscopic changes, especially in connection with the acute stages of glomerulonephritis.

Since the early 1900s, experimental research and clinical observations had suggested that glomerulonephritis was caused by two different immunological mechanisms: Anti-glomerular basement membrane glomerulonephritis and immune complex nephritis.

Using immunofluorescence staining, immunoglobulin and complement deposits in the glomeruli were shown. Each type of glomerulonephritis has its own staining pattern, i.e. linear or granular (26).

In 1956, Claus Brun became Senior Physician at the Central Laboratory (Clinical-chemical unit) at KH. He built a nephropathology laboratory, which, over the years, contributed a great deal of expertise in assessing light microscopic changes in glomerulonephritis. Claus Brun can only be described as the nestor of Danish nephropathology. A number of leading nephropathologists were trained in his unit.

One of them, **Svend Larsen** (1932-) has produced the most comprehensive series of immunofluorescence examinations of kidney biopsies in connection with glomerular diseases.

When he left school, Svend Larsen became an apprentice cabinetmaker. He finished his apprenticeship and received a silver medal in 1952. He became a master cabinetmaker in 1956, where he worked at and, for a time, managed, his father's carpentry business. He was keen to learn microscopy and attended a course at Danish Technological Institute in Copenhagen. He studied sections of beech and oak from several excellent trees.

This was not quite what he was looking for. He wanted to look at real human cells and tissue. He passed Upper Secondary School examinations at Ahms Kursus, a private college, in 1962 and graduated as a doctor at The University of Copenhagen in 1969.

(Incidentally, Svend Larsen and his wife, Rudy, were keen competition dancers in their youth. Svend wore out four or five tuxedos on the dance floor.)

Now he was a doctor and he wanted to microscope.

As an intern and later registrar at department 3 at KH, Svend Larsen became very interested in nephrology. At Claus Brun's unit, he had access to a wealth of histological and electronmicroscopic expertise. In 1973, with Jørgen Rygaard's support, Svend set up an advanced immunofluorescence laboratory, specialising in renal pathology. In 1981, his work resulted in a thesis, *Immune Deposits in Human Glomerulopathy* (27), which he defended with panache at Odense University (Figure 3.3.4.1).

He continued to study pathology, first at Gentofte and later at Herlev Hospital. In 1983, he became Senior Physician at the pathological institute and in 1986, Administrative Senior Physician at Københavns Amtssygehus (regional hospital, KAS) in Herlev. From 1987-2002, he was Professor of pathological anatomy and histology at the University of Copenhagen.

Over the years, he has produced innumerable scientific articles, most on nephropathology. In the context of the present publication, he has to be lauded for his sophisticated immunopathology techniques.

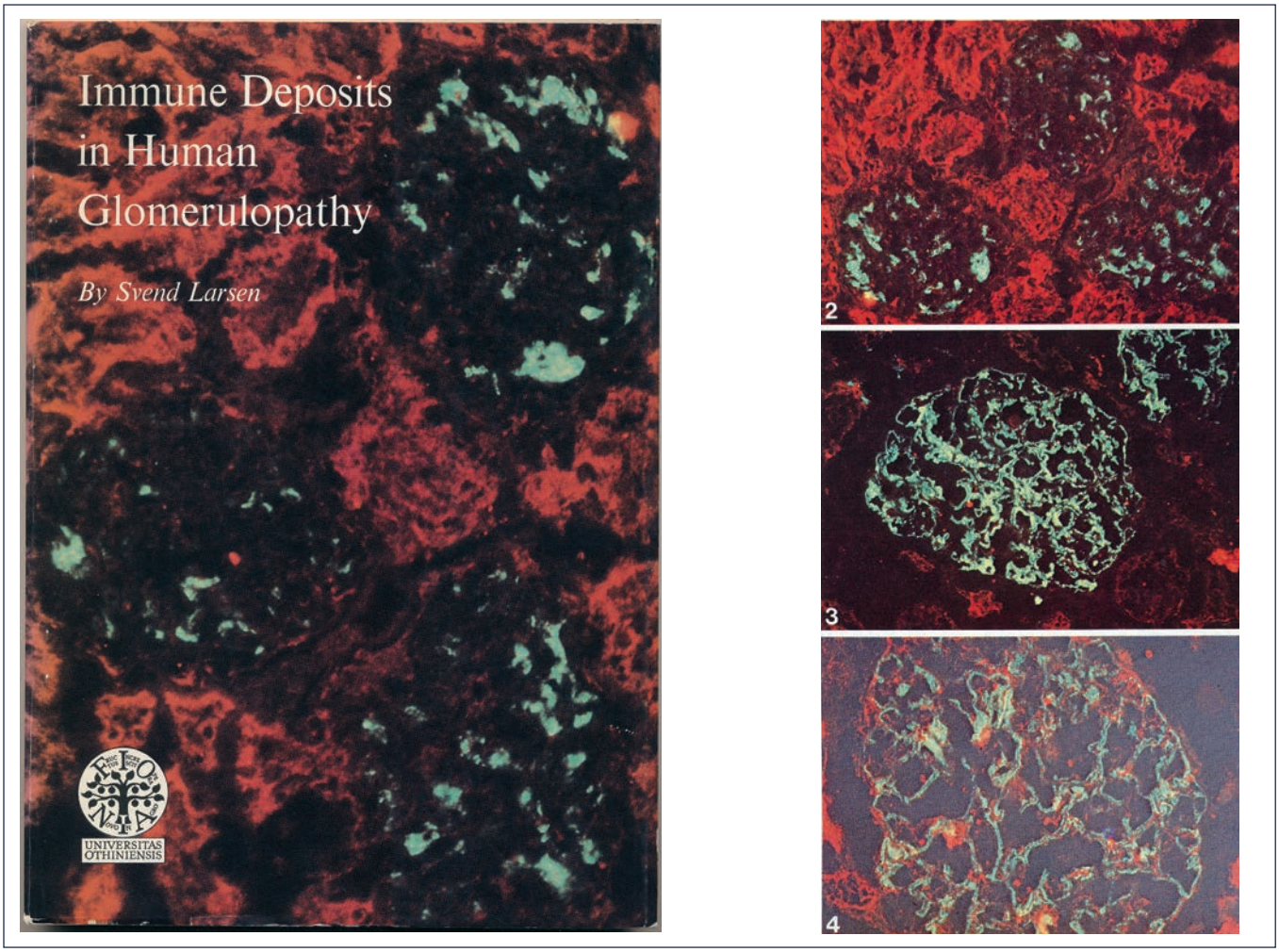

Figure 3.3.4.1. Svend Larsen's thesis from 1981. The thesis described immunofluorescence examinations of kidney biopsies from 290 patients with glomerulonephritis. Two types of staining reactions were shown: Linear and granular. Neither staining reaction was found to be related to histological changes, clinical symptoms or clinical processes. On right, granular deposits in glomeruli. **2** shows deposits of IgA in the mesangium. **3** shows deposits of complement C3 in the mesangium and basement membrane, whereas **4** shows deposits of IgG primarily in the basement membrane (28).

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3.4: HISTOCHEMISTRY AND DIAGNOSTIC HISTOPATHOLOGY

by Per Prætorius Clausen and Ole Nielsen

3.4.1: Diagnostic histopathology

The term "diagnostic histopathology" describes microscopic examination of tissue with a view to determining the nature of disease and prognosis as a part of the therapeutic strategy.

The first descriptions of diagnostic histopathological examinations were by **Bennet** in Edinburgh in 1845 (1) and **Donaldson** in Baltimore in 1853 (2). In both cases, the specimens were smears, not sections, which were not made until some years later. The advent of diagnostic histopathology is therefore also the advent of diagnostic cytology.

As mentioned earlier (Box 2.1), in 1852, **Adolph Hannover** was the the first scientist in Denmark to recommend a tissue biopsy in connection with tumour diagnostics (3). A period of just under 50 years was to elapse before the technique began to find widespread use. In the interim period, the vast majority of histopathological examinations was conducted in connection with autopsies. In the second half of the 19th century, progress in this speciality was rather slow in Denmark (4).

In 1900, **Johannes Fibiger** (1867-1928) was appointed professor of pathological anatomy. His appointment was a turning point, not only for teaching and research, but also for diagnostic tissue examinations.
Lægeforeningens Cancerkomité (Danish Physicians' Cancer Committee) was founded in 1905. Its purpose was to work to diagnose cancer as early as possible and thus reduce mortality. In 1908, statistics regarding cancer patients revealed that there were 1135 cases, of which 35% had been microscopically examined. In 1909, under Fibiger's leadership, microscopy became free of charge, which meant that poorer patients also had access to this service (5).

Microscopic examinations continued after Fibiger's death in 1928. In 1938, a total of 48,000 examinations were made. At this time, the examinations were made under the auspices of the three radium stations in Copenhagen, Odense and Aarhus. There were pathological institutes in all three cities and from this time onwards, examinations were performed only by specialists in pathological anatomy. Before 1938, at some locations, examinations were performed by clinical assistants in surgical units (4). In the next three decades, pathological institutes were set up all over the country.

The development in the number of microscopic examinations from the late 1930s until today is illustrated in Figure 3.4.1.1 Almost exponential growth is due partly to demographical conditions (population growth and changed age distribution) but especially to the increased use of percutaneous needle biopsy and biopsy taken in connection with endoscopic examinations.

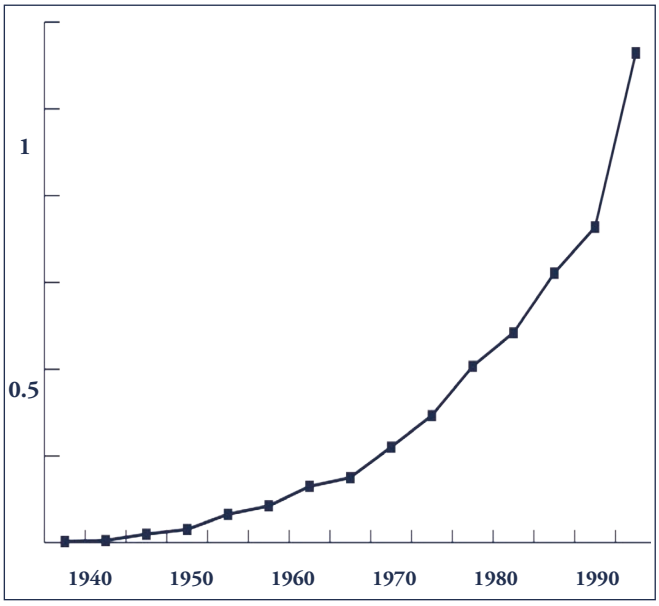

Figure 3.4.1.1. The number of requisitions (x 10^6) for microscopic histological examinations received at the pathology department at Odense University Hospital from the institute's foundation in 1937 until 1995. (6)

3.4.2: Staining methods in diagnostic histopathology

Haematoxylin and eosin stainings had already been introduced as separate histochemical staining techniques when, in 1876, **Wissowsky** described the use of both dyes in a double staining method (7). The combined haematoxylin-eosin (HE) staining has since without doubt been the method most often used in diagnostic histopathology. The majority of microscopic assessments and diagnoses made today are based exclusively on morphological assessment of HE-stained cell sections.

As described in Chapter 2, during the first half of the 20th century, a broad palette of staining methods (using special dyes) was used to illustrate tissue structures more specifically than could be achieved using HE staining. Many of these are still in use. These special dyes can be roughly categorised into the following groups: Dyes for nucleic acids, connective tissue components, carbohydrates, including mucosubstances, lipid staining techniques, staining for amyloid and staining techniques for endogenous amines, pigments, minerals and microorganisms, and staining of haematological cells.

The primary microscopic examination is usually made using an HE-stained cell section, after which, if necessary, a special staining is selected, depending on the pathoanatomical issue in question. The strength of these staining methods lies in that they highlight tissue structure and identify elements of certain substances. In diagnosing neoplasia, the "classical" special staining techniques offer limited information.

With the emergence of enzyme staining methods from 1940, it was hoped that the new staining methods would help to demonstrate the histogenesis and biology of neoplastic changes. Much research was conducted in the decades that followed (8), the results of which were, unfortunately, disappointing. It is only in connection with leukaemia classification that enzyme staining techniques have become standard methods (9).

Enzyme staining techniques have however become important in connection with the assessment of muscle biopsies and diagnostics and classification of myopathia (see Figure 2.2.1).

In the early days, immunohistochemical staining methods in the form of immunofluorescence staining were almost exclusively used in connection with diagnosing kidney and skin biopsies, especially in connection with immunologically determined conditions. These methods are still used (see 3.3).

The introduction of enzyme immunohistochemical methods used on formalin-fixed and paraffin-embedded tissue set off an almost revolutionary development in the use of immunohistochemistry to diagnose neoplastic transformations. Immunohistochemical characterisation of tumours is now an indispensable tool, used especially to classify poorly differentiated tumours (Figure 3.4.2.1), to determine the origin of metastasis (Figure 3.4.2.2) and to classify lymphomas (Figure 3.4.2.3) (10).

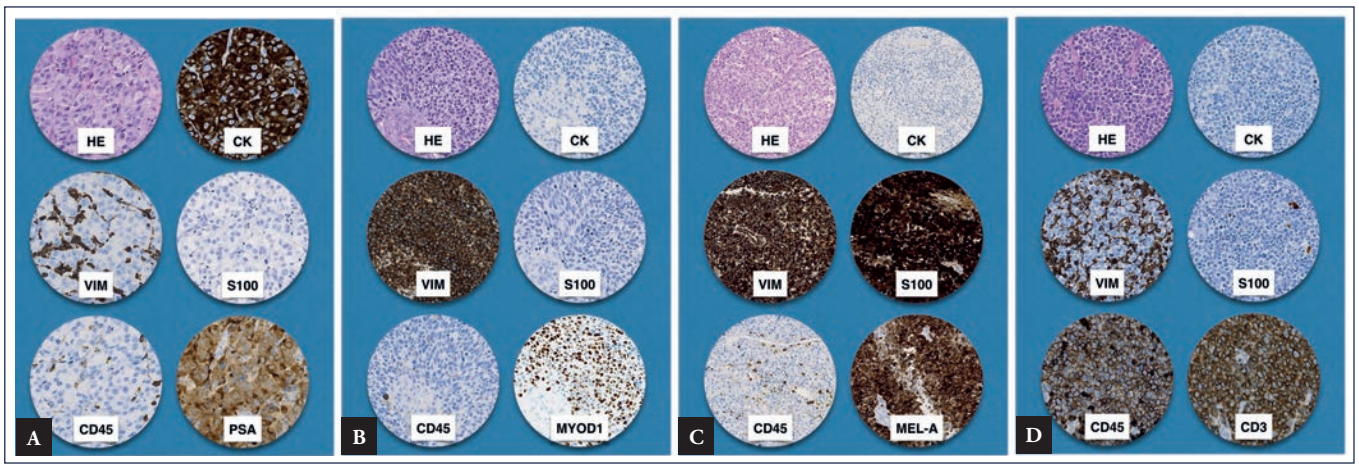

Figure 3.4.2.1. Staining of poorly differentiated tumours. Using a limited panel of antibodies, i.e. Cytokeratin (CK), Vimentin (VIM), S-100 and CD45, it is possible very accurately to classify poorly differentiated tumours, which can be difficult to classify on the basis of their morphological image, i.e. as this appears in a haematoxylin-eosin stained (HE) cell section. The primary antibody panel is supplemented with antibodies, which make classification incontestable. **A**: Prostate carcinoma (CK+, VIM-, S-100-, CD 45-) positive for prostate-specific antigen (PSA). B: Rhabdomyosarcoma (CK-, VIM+, S-100-, CD45-) positive for MyoD. C: Malignant melanoma (CK-, VIM+, S-100+, CD45-) positive for Melan-A. D: T-lymphoma (CK-, VIM-, S-100-, CD45+) positive for CD3. (Ole Nielsen)

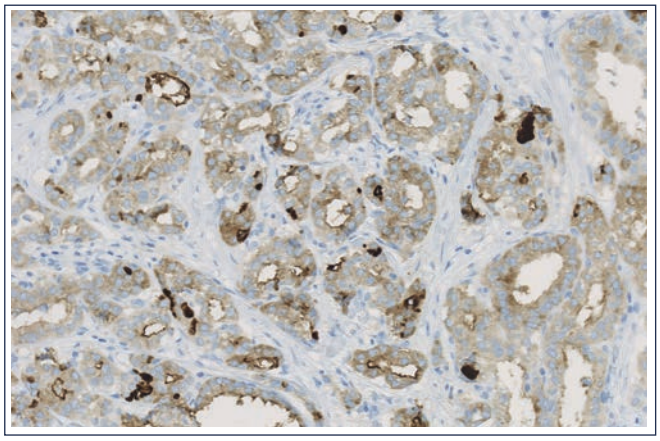

Figure 3.4.2.2. Metastasis from follicular thyroid carcinoma stained for thyroglobulin. (Ole Nielsen)

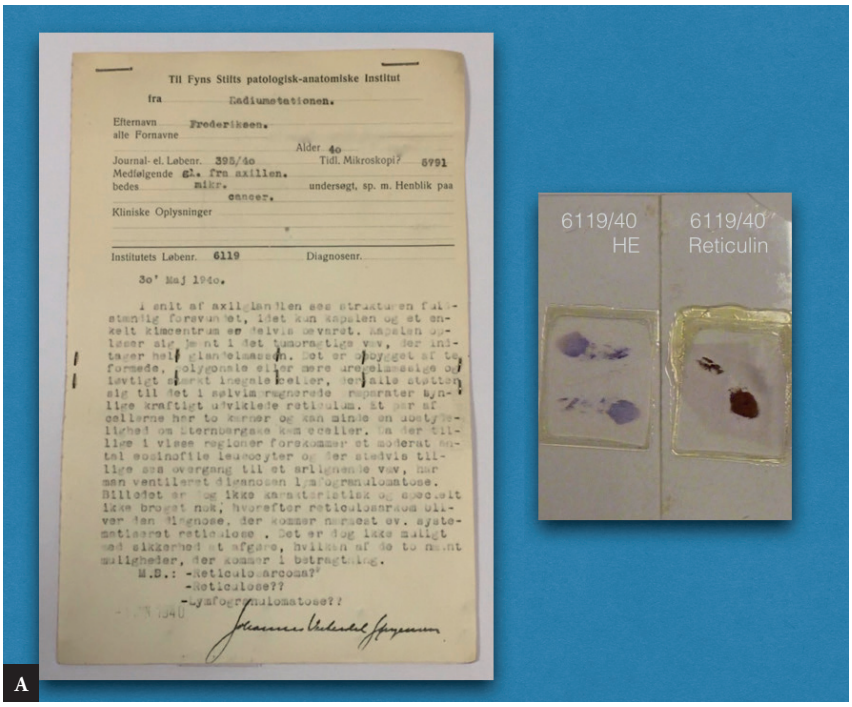

Figure 3.4.2.3. *The story of a malignant lymphoma*

These images speak louder than words regarding the importance of more recent immunohistochemical examinations to make more reliable lymphoma classification.

The pathological department was founded at Odense City Hospital in 1937. Three years later, the institute's prorector Johannes Vesterdal Jørgensen (1901-1976), received a lymph node, from which he described sections (**A**).

Seventy-six years later, new sections were cut from the original tissue blocks. Using a new HE stain and supplementary immunohistochemical stainings, it was now possible to make a precise classification of the lymphoma (Hodgkins Disease, lymphocyte depletion) as described by Birgitte Preiss.

The use of the different staining methods has changed over time and, in particular, the immunohistochemical methods are now relatively more prevalent (see Figure 3.4.2.4).

In the 1990s it was discovered that, in malignant tumours, there is often oncogene activation, suppressor gene deactivation and changes in DNA repair mechanisms. There was therefore a need to visualise these changes. Visualisation is normally achieved immunohistochemically by demonstrating the protein expression or by using *in situ* hybridization directly to demonstrate the genetic mutation. These investigations are now routine elements in characterising and classifying malignant tumours and therefore form the foundation for specific therapeutic strategies.

The use of immunohistochemical methods in combination with *in situ* hybridization in connection with pharmadiagnostic testing is described in section 3.5.

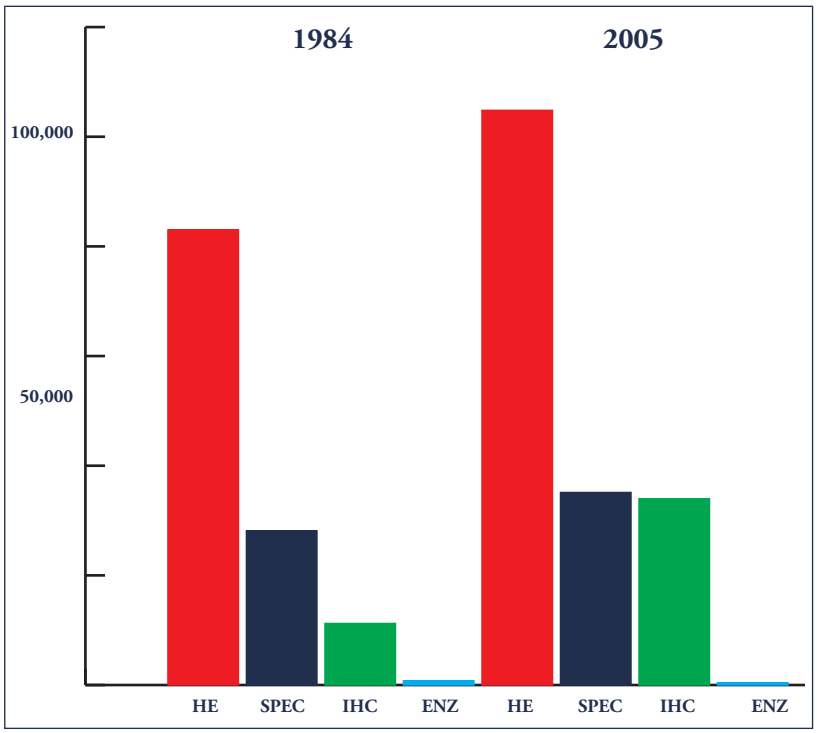

Figure 3.4.2.4. The number of stains per histochemical staining technique performed at the Pathological Department at Odense University Hospital (in 1984 and 2005, respectively) HE: Haematoxylin-eosin, SPEC: Special stains (see text), IHC: Immunohistochemical stainings, ENZ: Enzyme staining. (6)

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3.5: PHARMACODIAGNOSTIC TESTING AND RECENT MOLECULAR DIAGNOSTIC TECHNIQUES

by Jan Trøst Jørgensen

Within the past 10-15 years, a new type of predictive histochemical test has emerged, the so-called pharmaco-diagnostic assays, which are used in the treatment of certain cancers. This type of assay is used to identify the patients that most likely will benefit from treatment with a specific cancer drug. The development of these tests is based on a deep insight into the mechanism of the drug and the molecular conditions which must be present in the tumour for the drug to be effective. These assays are often referred to as "companion diagnostics". Pharmacodiagnostic assays are most often developed in a close, parallel process with the drug in question and therefore specifically linked to its use (1). Figure 3.5.1 shows a model of how a prospective development process between a drug and a pharmacodiagnostic assay could look (2).

Linking molecular testing and drugs development together was first seen in the 1970s in connection with the development of the anti-estrogen tamoxifen (Nolvadex®) for the treatment of women with breast cancer. This was, however, not a parallel development process. It was rather an investigation to explore oestrogen receptor status in some of the patients who took part in clinical trials (3). At that time, hormone receptor status (oestrogen/progesterone) was determined by means of a biochemical assay.

With the development of immunohistochemical assays, determining hormone receptor status became routine in diagnosing patients with breast cancer (Figure 3.5.2).

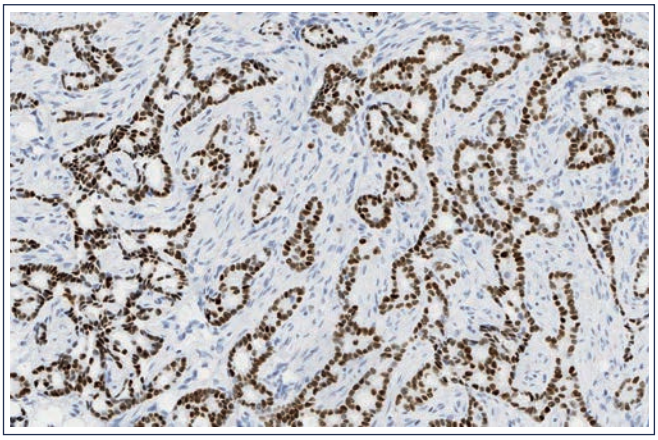

Figure 3.5.2 Staining of mamma carcinoma for oestrogen receptor, which shows a positive reaction in all nuclei. Immunoperoxidase staining with clone EP1 (Dako).

A positive hormone receptor status (especially for oestrogen) is a precondition for the effect of endocrine therapy with an anti-oestrogen or aromatase inhibitors (4, 5).

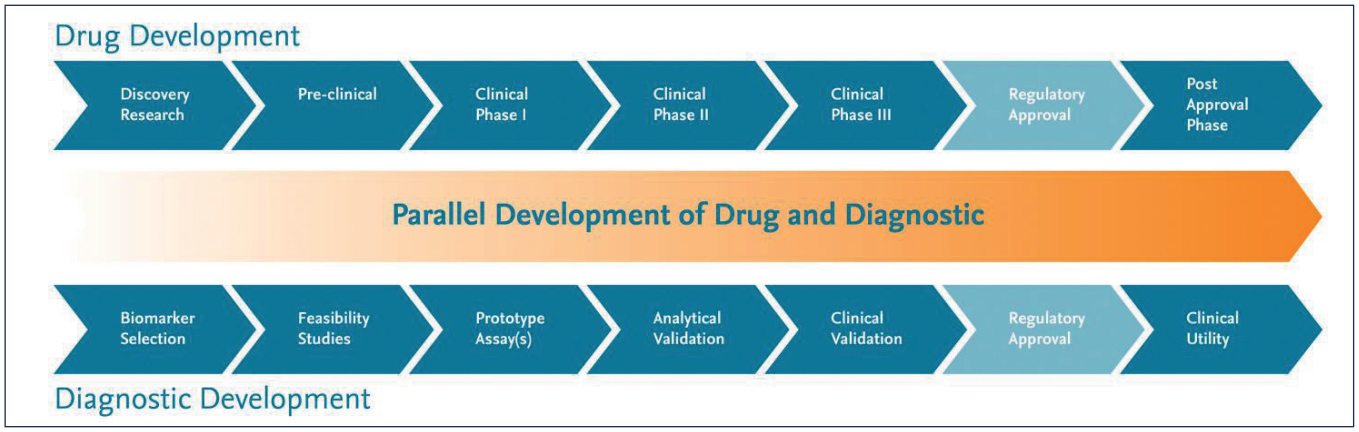

Figure 3.5.1 Parallel development process between a drug and a pharmacodiagnostic assay (2). This figure is printed by kind permission of Elsevier B.V. (Copyright Elsevier, 2015).

The first example of a parallel development of a drug and diagnostic assay was seen in the 1990s, when Genentech (an American biotech company) developed trastuzumab (Herceptin®) for treatment of women with advanced HER2-positive breast cancer. Along with the drug, an immunohistochemical assay to detect HER2 expression (HercepTest, Dako) was developed (5) (Figure 3.5.3).

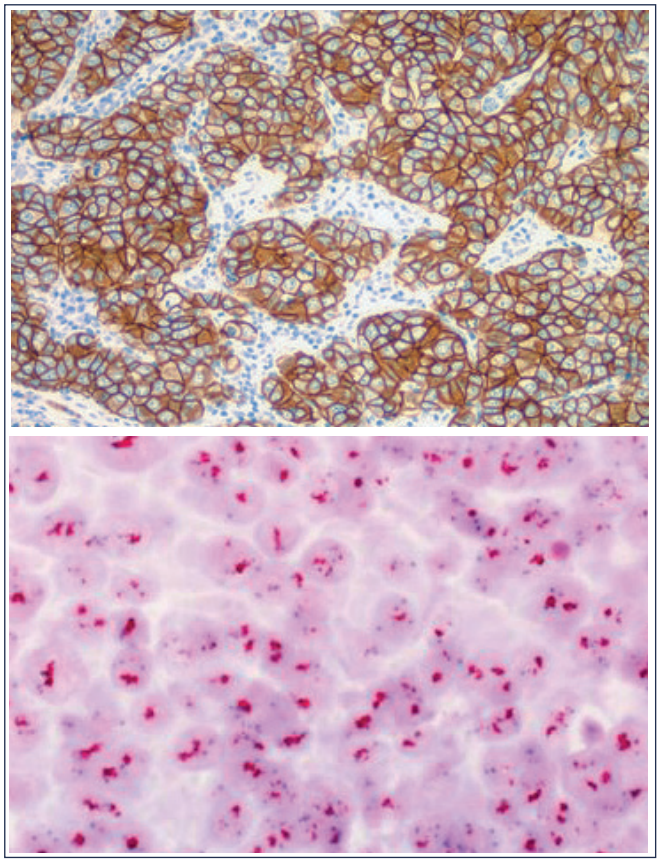

Figure 3.5.3 Staining of HER2 in mamma carcinoma. At the top, immunoperoxidase stain showing strong membrane-related positive reaction (HercepTest, Dako). At the bottom, *in situ* hybridization showing amplification of the HER2 gene (red spots) in the nuclei (*HER2* CISH pharmDx™ kit, Dako).

Since the turn of this century, a series of targeted cancer drugs have undergone a similar parallel development process. Table 1 lists some of these drugs with their respective pharmacodiagnostic assays (6). Common to all the assays listed in Table 1 is that they are based on different histochemical methods.

Test	Method	Medications
ALK Break Apart FISH Probe Kit	FISH	Crizotinib (Xalkori®)
ALK (D5F3) CDx Assay	IHC	Crizotinib (Xalkori®)
c-Kit pharmDx™ Kit	IHC	Imatinib (Glivec®)
EGFR pharmDx™ Kit	IHC	Cetuximab (Erbitux®) Panitumumab (Vectibix®)
ER/PR pharmDx™ Kit	IHC	Tamoxifen (Nolvadex®) Letrozol (Femar®) Anastrozol (Arimidex®) Exemestan (Aromasin®)
HercepTest	IHC	Trastuzumab (Herceptin®) Pertuzumab (Perjeta®) Ado-trastuzumab emtansine (Kadcyla®)
HER2 FISH pharmDx™ Kit	FISH	Trastuzumab (Herceptin®) Pertuzumab (Perjeta®) Ado-trastuzumab emtansine (Kadcyla®)
HER2 CISH pharmDx™ Kit	CISH	Trastuzumab (Herceptin®)
PD-L1 IHC 22C3 pharmDx	IHC	Pembrolizumab (Keytruda®)

FISH = Fluorescence in-situ hybridization
CISH = Chromogenic in-situ hybridization
IHC = Immunohistochemistry

Table 1. Pharmacodiagnostic assays.

In recent years, immunotherapy has gained acceptance as a treatment of cancer. For the PD-1 inhibitor pembrolizumab (Keytruda®), the expression of PD-L1 plays a role for the effect in non-small cell lung cancer patients.

In October 2015, the FDA approved pembrolizumab for the treatment of non-small cell lung cancer together with the immunohistochemical assay PD-L1 IHC 22C3 pharmDx (Dako) (7) (Figure 3.5.4).

In addition to protein expression, the number of gene copies and gene translocations, gene mutations can also impact the effect of a number of targeted cancer drugs. For example, a drug, such as vemurafenib (Zelboraf®), which is used to treat metastatic melanoma, only has effect in patients, whose tumours are positive for the *BRAFV600* mutation. To detect whether the patients are positive for mutation, the cobas® 4800 *BRAF V600* Mutation Test (Roche) is used. The test is a Polymerase Chain Reaction (PCR) assay (8).

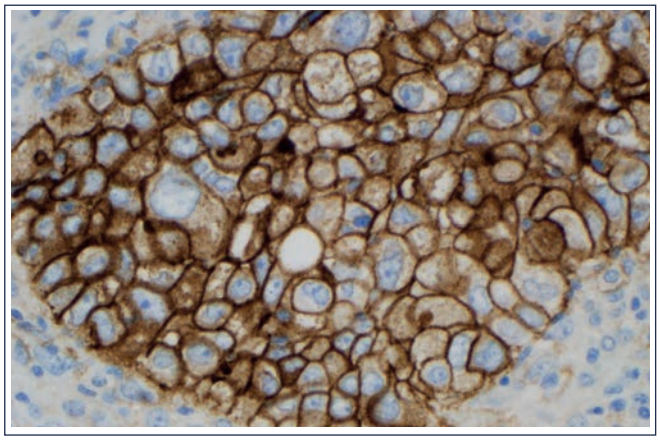

Figure 3.5.4 Non-small cell lung carcinoma (NSCLC) stained for PD-L1 with immunoperoxidase staining (PD-L1 IHC 22C3 pharmDx™, Dako).

In most cancers, there are multiple gene mutations and it makes sense to detect these using a single test. DNA sequencing, including the high-capacity "next-generation sequencing" (NGS) technology, could be an attractive solution. As yet there are no officially-approved pharmacodiagnostic assays based on NGS technology but this is probably only a question of time. Another area where both PCR and NGS technologies will undoubtedly play a role is in connection with analysing freely circulating tumour DNA (ctDNA). Research has shown that ctDNA detected in plasma can show whether the patient has developed resistance to an ongoing therapy (9). By measuring ctDNA, it will be possible to monitor for recurrence, which recently has been demonstrated in breast cancer (10).

In recent years, cancer is increasingly being classified according to its molecular characteristics. In this context, both conventional histochemical methods and the newer molecular diagnostic techniques will play a future role. The new molecular disease classifications will also play a decisive role in the development of new, more effective cancer drugs.

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CHAPTER 4: QUALITY CONTROL, CERTIFICATION AND STANDARDISATION

4.1: DANISH PARTICIPATION IN INTERNATIONAL QUALITY CONTROL

by Hans Oluf Lyon

4.1.1: The early days of international quality control

In line with the development of an overwhelming number of new dyes at the end of the 19th and beginning of the 20th century (see 2.1), it became clear that there was need somehow to secure the quality of the dyes so that users could rely on them to produce reproducible results.

The first steps towards assuring the quality of dyes were taken by **Carl Weigert** (1845-1904), who encouraged his pupil, **Georg Grübler**, to start systematically testing dyes to ensure that he achieved more or less uniform results. "The Grübler label" became a mark of quality. Dyes produced in Germany earned themselves what was tantamount to a worldwide monopoly.
After the outbreak of the First World War, access to German dyes was interrupted. This made it necessary to start producing dyes in the US, which was initially far from successful. The quality of the American dyes was so poor that most could not be used for bacteriological and histological purposes. They simply did not reach the same standard as "German quality".

When stocks of dyes produced by the Germans before the war were running dry, a commission of experts was set up in the US. The panel included experts within bacteriology, zoology, botany and laboratory work. In 1921, two conferences were hold. Both discussed standardising dyes. The **Commission on the Standardization of Biological Stains** then started systematically testing staining products using well-defined tests. In time, the laboratory work was financed by the income earned from selling certificate labels, which stain manufacturers could apply to their dye containers as a mark of quality.

During the Second World War, the work of the commission intensified and profits soared accordingly. In 1944, the commission was converted into a limited company and renamed **Biological Stain Commission (BSC)**. It still operates under this name today. In addition to regular testing and certification of dyes and stains, BSC also published books and journals. In 1925, **H. J. Conn**, the BSC Chairman at that time, published the first edition of the book, *Biological Stains*. This is now a standard reference book for the dyes and stains used in bacteriological and histological laboratories. The latest (10th) edition appeared in 2002 (1). In 1925, Conn also initiated the publication of *Stain Technology*, a journal, which publishes the results of BSC's work and the results of work at other laboratories. In 1991, the name of the journal changed to *Biotechnic & Histochemistry*. Since 2012, it has been published solely as an online publication.

4.1.2. How a DSCH Board member became involved in international quality control

At the VIth International Histochemistry and Cytochemistry Congress, held in Brighton, UK from 17-22 August 1980, **Hans Lyon** presented the results of analyses of dyes and stains manufactured by several producers, which were tested in line with Biological Stain Commission (BSC) protocols. The results for pyronin Y showed huge discrepancies with regard to the concentration of the dye in the samples tested (2) (Figure 4.1.2.1).

The presentation was welcomed, especially by the moderator, who was Professor of Anatomy **Dietrich Wittekind** from Freiburg, Germany. Wittekind had

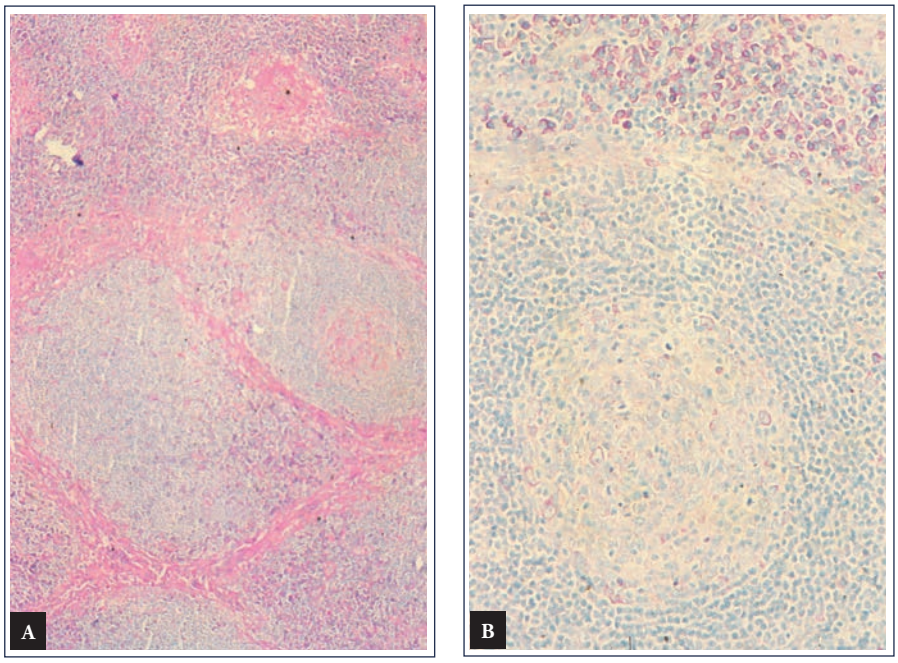

Figure 4.1.2.1. Methyl green-pyronin stains using two different makes of pyronin in 1978. A: Stain with pyronin from Gurr, showing distinctly unspecific staining in connective tissue, etc. B: Stain with pyronin from BDH with satisfactory staining of RNA. (Per P. Clausen)

worked with dyes and staining mechanisms for many years and is known for having demonstrated that the so-called *Romanowsky-Giemsa effect* is solely due to a reaction between the tissue and cell elements and the dyes, azure B and eosin (3).

After the lecture, Hans Lyon and Dietrich Wittekind had a good chat about the importance of using pure dyes in biological testing. As a result, Hans Lyon was invited to Freiburg to take part in a group, all of whose members were similarly interested in dyestuff purity. The group included **Erik Schulte** (Germany), **Andreas De Leenheer** (The Netherlands) and **Richard Horobin** and **Michael Barer** (both from the UK).

4.1.3: European standardisation

In 1987, the group was recognised as a working committee under the *European Committee for Clinical Laboratory Standards* (ECCLS). The working committee published a number of articles on standardisation of specific dyes in *Histochemical Journal* (4).

In the early 1990s, Wittekind and Schulte took the initiative via the German standards body DIN and Lyon via Danish Standards (DS) to transfer the group's work to the *European Committee for Standardization* (CEN), which takes care of standardisation within the EU and EFTA. In the wake of much (and, at times, difficult) negotiations and with invaluable assistance from many quarters, including **Lars Vejlens** in Sweden, the group succeeded in getting CEN to approve the group's standards as EN ISO 19001 under the auspices of the CEN technical committee : *TC 140, In vitro diagnostic devices.*

4.1.4: International standardisation

Shortly after, the *International Organization for Standardization (ISO),* which is an organisation of 162 member states that promotes and coordinates uniform industrial standards, set up a new technical committee: *ISO/TC 212: Clinical tests.* Hans Lyon was authorised by *DS* to represent Denmark on *ISO/TC 212*. The CEN document was converted and approved as *EN-ISO 19001* in 2002. The document is intended for reagent manufacturers and deals with reagents and selected procedures. It contains information regarding purity and the use of dyes in in vitro diagnostic tests. It was subsequently revised and approved as *EN ISO 19001:2014*.

This document was then supplemented in 2015 with a document intended for users, approved as *ISO/TS 17459:2015, Medical Laboratories -- Reagents used for staining biological material - Guidance for users.*

In November 2015, at a meeting of *ISO/TC 212*, a new project was proposed: *Molecular in vitro diagnostic examinations - Specifications for pre-examination processes for FFPE (Formalin Fixed Paraffin Embedded) tissue - Part 4: Diagnostic Staining.* Earlier phases of the project are Part 1 (DNA), Part 2 (RNA) and Part 3 (Proteins).

In 1998, Wittekind was elected a member of the BSC Board. He proposed that Schulte and Lyon presented some of the work they had done (described above) at BSC's annual meeting. In 2002, Lyon was elected a member of the board and has subsequently contributed e.g. to BSC publications (1.5-16).

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4.2: THE WEST DANISH ERFA GROUP AND EXTERNAL QUALITY CONTROL
by Ole Nielsen

4.2.1: The first investigations on method parameters

In 1974, Taylor and Burns (1) were the first to describe the use of the immunoperoxidase technique to demonstrate antigens in formalin-fixed, paraffin-embedded tissue sections. Then, in 1976, **Huang** et al (2) supplemented with their discovery of proteolytic enzymes' demasking effect. These discoveries forged a path for the use of immunohistochemical (IHC) methods in routine pathoanatomical diagnostics. However, in the early years, the supply of antibodies relevant to diagnostics was limited. In the early 1980s, the use of immunoperoxidase gathered momentum – also on paraffin-embedded sections.

When Hvidovre Hospital opened in 1976, pathologist **Per Prætorius Clausen** (PPC), then a registrar, set up a small IHC laboratory with two medical laboratory assistants, **Niels Johansen** and **Annelise Petersen**. In its daily work, the team soon recognised the impact of fixation on the staining reactions. By the late 1970s, working this time with **Marianne Jacobsen** and **Grete Krag Jacobsen** at the pathology department at Herlev Hospital, PPC conducted a battery of systematic investigations into the significance of fixation for the detection of a large number of antigens (3,4).

In the mid-1980s, in connection with his appointment as senior physician at the pathology department at Odense University Hospital (OUH) and working closely with a medical lab tutor, later project coordinator, **Ole Nielsen** (ON), PPC set up an IHC laboratory. The laboratory embarked on a comprehensive systematic review and investigation into the significance to the final staining result of a large number of important method parameters. The parameters investigated included the choice of fixative, fixation time, proteolytic pretreatment, incubation times for the primary antibody, wash buffer composition and washing times after antibody incubation, and a variety of visualisation methods (detection systems). The results of these many investigations were not published but, in the early 1990s, they formed the basis for a series of IHC workshops held by DSCH. The first workshop, held in January 1991 at the Royal Danish Veterinary and Agricultural College (KVL) in Frederiksberg (Copenhagen) was entitled: *Workshop in applied immunohistochemistry. Methods and diagnostics*. The diagnostic part of the workshop was given by **Mogens Vyberg**, while, building on several years of investigating methods, PPC and ON – mainly using microscopy exercises – "demonstrated how immunohistochemical staining reactions depend on the type of tissue preparation, fixation, reagents and techniques used". The workshop was a success. It was repeated several times in subsequent years. In the period 1991-2012, similar courses were held by Danske Bioanalytikere (Danish union of medical laboratory scientists, dbio).

4.2.2: IHC-ERFA

In 1990, the same year as the DSCH workshop was planned, the pathology unit at OUH took the initiative to set up an IHC-ERFA group for pathology departments in the western half of Denmark. The initiative was sponsored by Dako. The objective was to create a forum, at which the laboratories could swap experiences (the Danish word for "experience" is "ERFAring") related to IHC and immunohistochemical method parameters. A similar ERFA group was set up on Zealand (East Denmark). In the initial years, pathologists and biomedical laboratory scientists in West Denmark met 2-3 times a year at an ERFA meeting, at which IHC-related issues were presented and discussed. The Zealand ERFA group dissolved itself about ten years ago. Since then the West Danish ERFA group has expanded to include all the Danish pathology units and similar laboratories with an interest in IHC.

There is now an annual meeting, always on the first Tuesday in October and always at Lillebælt Sygehus, a hospital in Vejle, at which **Judith Jensen**, who tutors trainee medical laboratory scientists, and her colleagues make sure that the meeting is enjoyable for everyone. The meeting agenda is planned partly on the basis of ERFA meetings and partly

on input from the ERFA steering committee, which, over the years, has had many enthusiastic members (none mentioned by name – and none forgotten). Professor **Stephen Hamilton** has been the inspirational moderator at these meetings for more than 15 years. After the "merger" with the Zealand group, the goal of the ERFA meetings is unchanged; by sharing experiences to help maintain high standards of uniform quality in diagnostic histochemistry at Danish pathology units.

The West Danish ERFA group celebrated its silver jubilee in 2015. In the group's 25-year history, there were major breakthroughs in immunohistochemistry, which were decisive for pathoanatomical diagnostics. In 1991 (5), **Shi** et al discovered heat-induced epitope retrieval (HIER), which significantly expanded the boundaries for what it is possible to achieve using a paraffin-embedded section. In 1995, the work of **Katherine Knight** and her colleagues (6) created the basis for production of monoclonal rabbit antibodies. In 1998, Dako was the first to launch a polymer-based detection system. EnVision™, as the system was named, eradicated the difficulties associated with unspecific background staining in connection with endogenous biotin, which was (and still is) a problem inherent to avidin-biotin-based detection systems (ABC and LSAB systems). The 1990s was also the decade, in which the first semi-automated immunohistochemical stainers arrived at Danish pathology units, improving IHC stain reproducibility and the individual laboratory's staining capacity accordingly. All of these breakthroughs have of course made their mark on ERFA meeting agendas over the years and many pathologists and hospital laboratory scientists have contributed know-how (Figure 4.2.2.1).

In addition to creating a forum for exchanging IHC-related experience, there has always been a strong focus on external quality control. In the period 1990-2001, different models for external quality control were tested. The most ambitious project was launched in 1992. All 13 pathology units in West Denmark took part. The external quality study included 57 pathologists at 13 different units. Each was sent 12 unstained paraffin-embedded sections from a multi-tissue control block (Figure 4.2.2.2) containing tissue from 13 different tumours, which they were asked to diagnose using the laboratory's antibody

Figure 4.2.2.1. Examples of presentations held at meetings of the (West) Danish ERFA group.

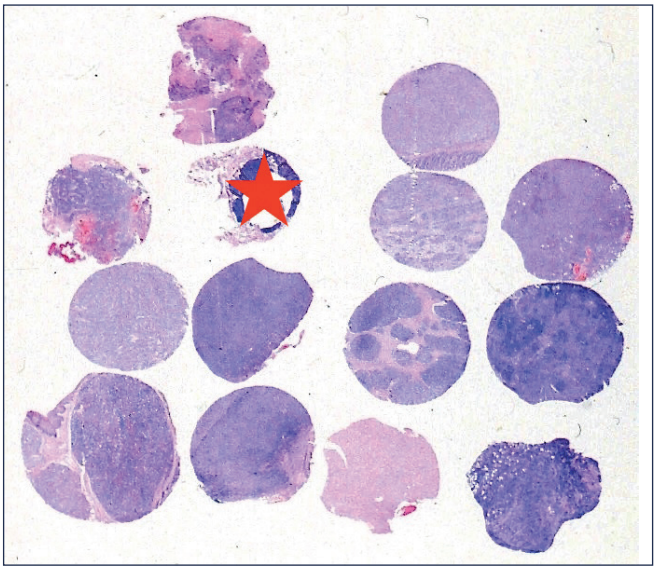

Figure 4.2.2.2. HE section of multi-tissue control block with 13 different tumours.

panel of choice. The antibody panels, methods etc. were collected with the diagnoses and analysed.

This study was not only an opportunity to assess the significance of a number of technical parameters for the diagnostic result, but also some of the interpretive aspects of the IHC analyses. It was interesting to note that this quality study showed that "the most important parameter for a correct diagnosis is an appropriate choice of antibody panels and the interpretation of the IHC stains" (7).

4.2.3: From IHC-ERFA to NordiQC

Until 2001, the ERFA group conducted a series of more classical external quality studies of the laboratories' IHC stains. These tests mainly involved sending a number of unstained sections of paraffin-embedded multi-tissue control blocks to the participating laboratories. The laboratories then had to stain a series of diagnostically relevant antigens, on which the group had agreed in advance. Along with staining procedure data, the stains were returned to the working group, who assessed and compared the quality of the sections received. The results were then presented at the next ERFA meeting, where the laboratories could see examples of the antibodies/methods that gave the best results – and those which were not quite as good (Figure 4.2.3.1). The pathology units in Aarhus, Odense or Aalborg were responsible for these so-called "antibody assessments" (see Section 4.3). In Aalborg, the task was primarily assigned to **Mogens Vyberg** and **Søren Nielsen.**

Having "practised" for some years within the framework of the West Danish ERFA group, it is widely known that Vyberg and Nielsen helped to start the well-renowned, independent scientific organisation, NordiQC, which provides external immunohistochemical quality controls at the international level (see 4.3). Since the advent of NordiQC, the ERFA group has succeeded in maintaining close contacts with the organisation.

Since 2004, NordiQC has been a regular item on the agenda at ERFA meetings, i.e. Vyberg and Nielsen have presented the results of the previous 12 months' NordiQC assessments.

At the end of 2015, the ERFA group is still going strong. The annual meeting attracted about 110 highly motivated participants to Vejle from all parts of the country. There is every indication that IHC-ERFA meetings will continue to be an important forum for sharing experience within diagnostic immunohistochemistry.

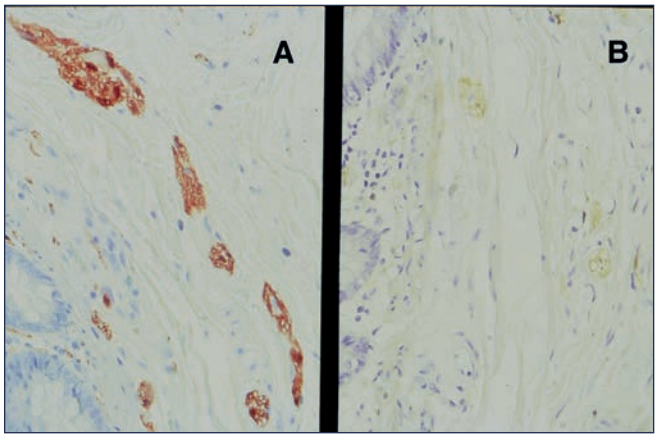

Figure 4.2.3.1. Examples of S100 stains from the ERFA group's first antibody assessment in 1990. Lab "**A**" achieves an expected strong S100 reaction in the peripheral nerves (Schwann cells) of the colon. Lab "**B**" fails to achieve an acceptable result in the same task.

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4.3: NORDIQC AND EXTERNAL QUALITY CONTROL IN IMMUNOHISTOCHEMISTRY

by Søren Nielsen and Mogens Vyberg

4.3.1: The build-up to NordiQC

During the 1980s, Dako in Glostrup started collaboration with several immuno-laboratories about tests of antibodies and reagents, also at the Institute of Pathology, Aalborg Hospital, where Mogens Vyberg in 1988 was appointed as senior physician and given medical responsibility for immunohistochemistry (IHC). At the same time, with the foundation of the West Danish IHC ERFA group (see section 4.2.), there were early signs of interest in external quality assessment (EQA) of IHC. Inspired partly by the foundation of the ERFA group, Dako Norden took the initiative in 1999 to organise a meeting on the periphery of the European Congress of Pathology in Barcelona, at which representatives of nine Scandinavian pathology units, including Aalborg, met to discuss the setting-up of a Scandinavian quality assurance project in line with United Kingdom National External Quality Assessment for Immunocytochemistry (UK NEQAS ICC). A series of ring trials, in which up to 55 Scandinavian laboratories took part, revealed unacceptably wide variations in staining results even for well-characterised immune markers. The lessons learned from these trials both showed the need and gave the basic experience to set up a permanent organisation, Nordic Immunohistochemical Quality Control (NordiQC), with effect from 01.01.2003 (Figure 4.3.1.1).

Figure 4.3.1.1. The NordiQC logo – a polar bear footprint, showing the antigen antibody reaction as the dot over the i.

4.3.2: NordiQC is founded

From the start, NordiQC was led by a core group comprising a pathologist representative from each of the Nordic countries, Denmark (**Mogens Vyberg**), Finland (**Heikki Helin**, from 2011, **Ari Ristimäki**), Norway (**Emina Torlakovic**, from 2003 **Bjørn Risberg**, and from 2009 **Jan Klos**) and Sweden (**Tomas Seidal**, from 2012 **Viktoria Gaspar**). The organisation was entrenched at the Institute of Pathology in Aalborg, with Biomedical Laboratory Scientist **Søren Nielsen** as the technical manager.

Since its conception, NordiQC's primary focus has been to define and identify optimal staining results, identify the antibodies, protocols and platforms that seem to be important for optimal versus inadequate staining results, identify control materials to assess quality and reproducibility and to describe all of these conditions on its website and to provide individual recommendations for the laboratories (see below).

The core group agreed on three annual rounds of stain quality testing for 5-6 widely used immune markers based on paraffin-embedded multi-tissue blocks produced for the purpose. As a precondition for participation, the laboratories have always had to report central protocol parameters (a total of about 50) including antibodies/clones, preparation methods, detection kits and staining platforms. Together with Søren Nielsen, the core group has constituted an assessor group, which has since joined forces with Project Coordinator **Ole Nielsen**, Odense, and Biomedical Laboratory Scientist **Michael Bzorek**, Næstved. The group reviews all the stains received in plenum using a multi-head microscope. Each stain is awarded a score (optimal, good, borderline or poor) and the results are sent by email to the participating laboratories. An unsatisfactory result (borderline or poor) is always accompanied by an explanation and recommendations regarding e.g possible change of the antibody and/or other key protocol parameters.

In response to a specific need for consistent assessment of the detection of predictive markers (oestrogen receptor and HER2), a separate assessment module has been set up for IHC in breast pathology. Initially, the Danish participants in this module were senior physician **Birgitte Bruun Rasmussen** (Roskilde) and since 2010 Senior Physician **Vibeke Jensen** (Aarhus). Furthermore, a separate module for *in situ* hybridization for HER2 has been established, in which Ole Nielsen, Michael Bzorek and Senior Physician **Anne-Vibeke Lænkholm** (RH, later Slagelse Sygehus (hospital)) are associated.

4.3.3: Publishing results in NordiQC

All the general results of the quality assessments are published on NordiQC's website. The results include a review of the key protocol parameters as (based on systematic analysis of IHC results and the methods used) these are regarded as having potential impact on the staining results for individual immune markers. For each immune marker, NordiQC has therefore identified and described the most reliable antibodies and "best practise" parameters on the most widespread platforms. The website also provides examples of recommended control materials and images of expected reaction patterns in these, and examples of optimal and unsatisfactory stain reactions in circulating tissue sections (Figure 4.3.3.1).

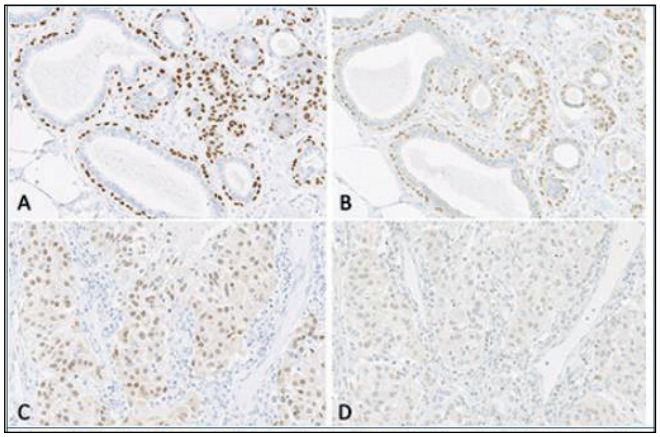

Figure 4.3.3.1. Illustration showing the difference between optimal and suboptimal SOX10 staining. Left: optimal reaction in mamma tissue (top) and malignant melanoma (bottom). See http://www.nordiqc.org/Run-45-B20-H8/Assessment/Run45_SOX10.pdf.

The website is open to everyone, i.e. all laboratories and companies can make use of the data.

In 2003, 70 laboratories in the Nordic countries took part in NordiQC (i.e. almost all of those who performed IHC of any significance). Laboratories outside the Nordic region have since begun to apply to join. In 2015, there were more than 700 participating laboratories in more than 80 countries. Until 2015, NordiQC has performed 45 assessment rounds in the general module, 20 rounds in the breast cancer module and 8 rounds in the HER2 ISH module, and all together 20 rounds for about 90 different IHC markers have been performed. The total number of immuno-stained sections assessed until 2015 was just over 30,000 (corresponding to about 150,000 tissue sections).

About 30% of all immuno-stainings in the general module are assessed as unsatisfactory. The same applies to about 20% of stainings in the breast cancer module. Among about 9,000 unsatisfactory stainings, about 90% are characterised as too weak or as false-negative. In the vast majority of cases, the assessors have been able to indicate the one or several most important causes (poor antibodies, poorly calibrated ready-to-use product, an antibody sensitive to a specific platform, incorrect antibody dilution, inadequate demasking and insensitive visualisation system).

Individual guidance and protocol analyses seem to have had a positive effect. Laboratories, which have taken part in several rounds, have a 20 percent higher pass rate than laboratories taking part for the first time. The results of laboratories, which, in response to an inadequate result, change their procedures in line with NordiQC's recommendations improve by about 50 percent compared to laboratories which do not implement the recommendations. The results from 14 HER2 rounds in the mammacancer module reveal that laboratories that develop their own HER2 protocols (presumably to save money) produce significantly poorer results than laboratories, which use CE-marked and FDA-approved kits. In addition to the human cost, this inflicts huge losses on the healthcare sector. These conclusions have been published. NordiQC engages in an ongoing dialogue with companies which continue to sell poor antibodies or provide misleading protocol instructions. In isolated cases, the organisation has been forced to notify the FDA in order to get a response.

In Denmark, thanks to ERFA meetings and numerous local training courses, primarily initiated by Ole Nielsen (Odense), there is a long-standing tradition for systematically reviewing immunochemical opportunities and threats, the general effect of which is a positive knock-on effect on the Danish laboratories' NordiQC pass rate compared to the other Nordic countries. The figures (below) show this clearly.

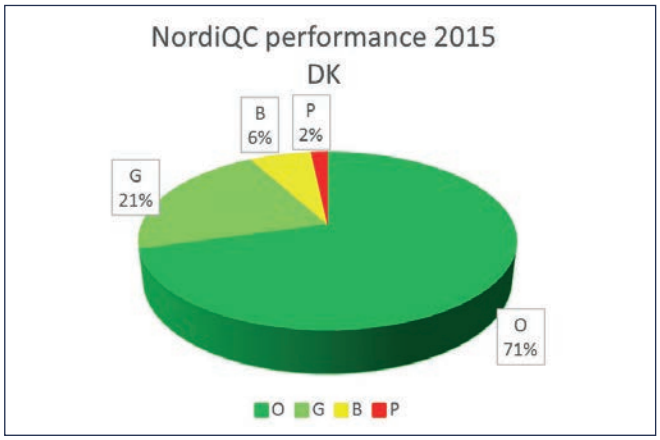

Figure 4.3.3.2a. Distribution of scores for Danish laboratories in NordiQC "General module" 2015.

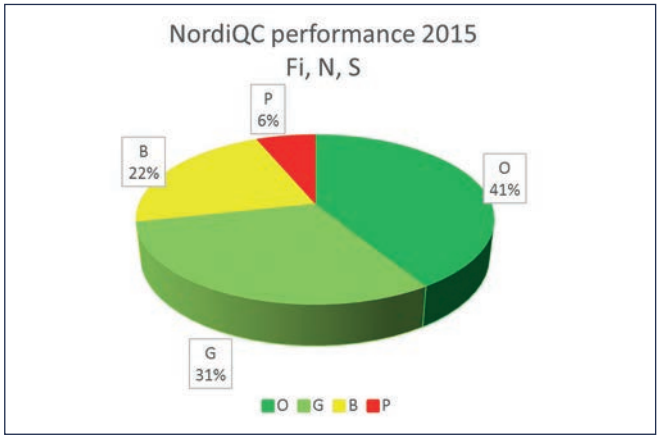

Figure 4.3.3.2b. Distribution of scores for other Scandinavian laboratories in NordiQC "General module" 2015. (O: Optimal, G: Good, B: Borderline, P: Poor).

At the time of writing, NordiQC's permanent staff (all employed at Aalborg University Hospital) comprises Scheme Director Mogens Vyberg, Scheme Manager Søren Nielsen and Scheme Organiser **Rasmus Røge**, two biomedical laboratory scientists (**Lise Emanuelsen** and **Jesper Lund Lauridsen**) and two medical secretaries (**Maria Lund Nielsen** and **Lilli Guldager Malaca)**. Everyone on the team is a part-time employee of NordiQC.

4.3.4: NordiQC training courses and scientific publications

Over the years, NordiQC has arranged or contributed to meetings and short courses in the Nordic countries, e.g. in connection with pathology societies' annual meetings and company users' gatherings. Outside Scandinavia, one of more of the NordiQC team of pathologists (Søren Nielsen, Jan Klos, Rasmus Røge and Mogens Vyberg) have lectured in many countries, including Poland, Italy, USA, Canada, Russia, China and Australia. In Aalborg since 2008, three-day workshops have been held for biomedical laboratory scientists, natural scientists and pathologists. The workshops focus on the use of immune markers in tumour diagnostics, and include a review of pre-analytical, analytical and post-analytical parameters, protocol optimisation and the use of controls, as well as technical and diagnostic pitfalls. Most participants are from Scandinavia but some have come from as far afield as North and South America, Asia and Australia. There are only 50 places and there has been a waiting list of up to two years. Since 2014, the Aalborg workshops (led by Søren Nielsen) have been aimed more directly at biomedical laboratory scientists as there are now parallel annual workshops (led by Jan Klos) in Krakow, targeted directly at pathologists. International IHC Conferences were held in Aalborg in 2013 and 2015. Each attracted about 300 attendees. The next conference will be in 2017.

Many articles on tests of specific immune markers and articles presenting general IHC guidelines have been published. The articles are based on the huge volume of data generated in NordiQC. Some of the articles are published in cooperation with the IHC EQA organisations in Canada, UK and Australia (see reference list below).

In 2015, inspired by the European Society of Pathology (ESP), an umbrella organisation, *International Quality Network Pathology* (IQN Path), was formed. The founding bodies are NordiQC, UK NEQAS ICC and seven other international organisations, all of which work with quality control within IHC and molecular biology. The organisation is registered in Luxembourg. The president is **Han van Krieken**, The Netherlands. **Jacqueline Hall**, UK, is the director. The objective of IQN Path is to create a forum for development of international guidelines for pathoanatomical biomarker testing. IQN Path works with other international bodies, including WHO. In 2016, NordiQC co-founded a new scientific society, the *International Society for Immunohistochemistry and Molecular Morphology* (ISIMM). **Clive R. Taylor** is the new society's first president. This society's primary task is to promote IHC research and education.

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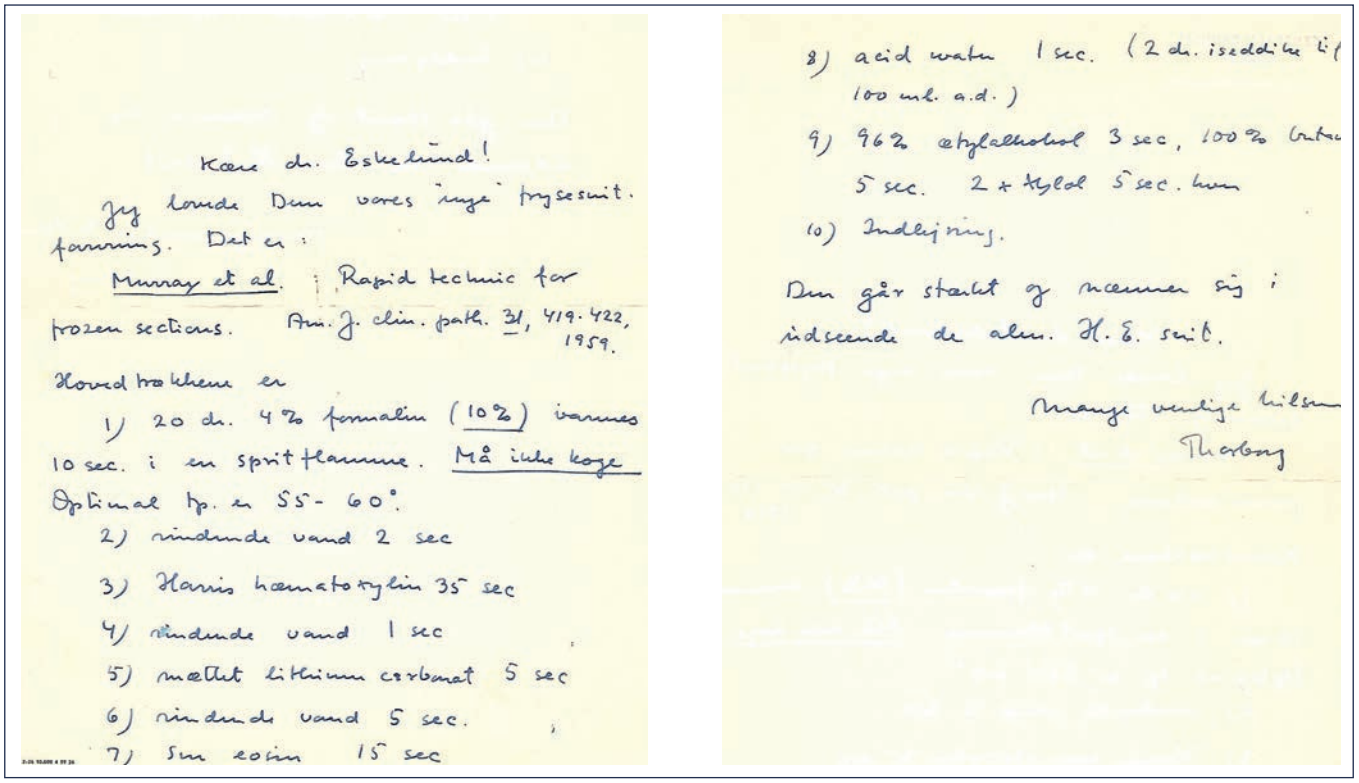
CHAPTER 5: EDUCATION AND POSTGRADUATE TRAINING IN HISTOCHEMISTRY IN DENMARK

by Inger Lindebo Holm, Anne Palle Andersen and Hans Oluf Lyon

5.1: THE ROAD TO A BASIC TRAINING FOR BIOMEDICAL LABORATORY SCIENTISTS (HISTO)

Until the 1960s, laboratories' sharing histochemical knowledge and experience took most often place by personal exchanges (Figure 5.1.1).

Young registrars originally performed analytical work. In the period 1920-1950, analytical work was gradually transferred to "laboratory technicians" who received on-the-job training. As the analysis repertoire grew and de facto clinical laboratory units were set up, there arose a need for formal and standardised training. During the 1950s, there was a variety of Biomedical Laboratory Scientist training courses (most with clinical chemical focus). The first formal course of training as a Biomedical



Kære dr. Eskelund!

Jeg lovede Dem vores ingø frysesnit-farvning. Det er:

Murray et al.: Rapid technic for frozen sections. Am. J. clin. path. 31, 419-422, 1959.

Hovedtrækkene er

1) 20 dr. 4% formalin (10%) varmes 10 sec. i en spritflamme. Må ikke koge

Optimal tp. er 55-60°.

2) rindende vand 2 sec
3) Harris hæmatoxylin 35 sec
4) rindende vand 1 sec
5) mættet lithium carbonat 5 sec
6) rindende vand 5 sec.
7) Sun eosin 15 sec
8) acid water 1 sec. (2 dr. iseddike i 1 l 100 ml. a.d.)
9) 96% ethylalkohol 3 sec, 100% [illegible] 5 sec. 2 × tylol 5 sec. tørr
10) Indlejring.

Den går stærkt og nemmen sig i sidstnævnte de alm. H. & E. snit.

Mange venlige hilsner
Thorborg

Figure 5.1.1 Danish laboratories exchanged – and continue to exchange – experience and expertise mostly via personal contact. Here prosector J. V. Thorborg in Randers shares a trick of his trade (rapid staining of cryosections) with Associate Professor Viggo Eskelund (see Figure 2.1.11) at Rigshospitalet in the late 1950s. (Found in a staining protocol book that belonged to Viggo Eskelund's laboratory assistant, Kit Brest-Nielsen.)

Laboratory Scientist (clinical chemistry) was offered in 1958. In 1960, the authorities began to consider a course of training leading to a Histo Medical Laboratory Scientist qualification and, during the 1960s, Landsforeningen af Hospitalslaboranter (LaH, now The Danish Association of Biomedical Laboratory Scientists, dbio) offered training courses for hospital Biomedical Laboratory Scientists working as histo laboratory assistants.

The component parts of this world-class histochemical training were: Professional expertise, enthusiasm, good coordination skills and a good cooperation with the laboratories/clinic.

The basic professional histochemistry training outside the laboratories was launched by Associate Professor **Knud Jørgen Pedersen** at the Department of Zoology at the University of Copenhagen (KU). K. J. Pedersen researched into and taught tissue biology and was therefore both a keenly interested and very erudite histochemist. In the early 1960s, he set up optional courses at KU on "histological histochemical methodology". These courses were later signally important for tutors who taught histochemistry as part of Medical and Biomedical Laboratory Scientist training courses.

On Pedersen's course, students crammed all the usual staining methods in theory and practice. After a couple of years, **Poul Prentø** MA, joined as tutor. The tissue studied was not actually human tissue. It was from rabbits, mice, rats and worms. The histological/histochemical expertise was, however, superlative. A registrar, **Hans Lyon** began to show an interest in histological technique and histochemistry. Lyon met K.J. Pedersen and Poul Prentø on their histology/histochemistry methodology course in 1965. Within a few years, a civil engineer, **Anne Palle Andersen** (study coordinator for histo training 1967-1989) signed up for the course. In 1973, **Inger Lindebo Holm** M.Sc. (study coordinator/department manager 1989-1995) did the same.

In 1966, Anne Palle Andersen was recruited to Hospitalslaborantskolen (school for Biomedical Laboratory Scientists). She was responsible for the histo Biomedical Laboratory Scientist training course from 1967. Her recruitment to this position was fortuitous. The school wished to employ a physician but the ministry responsible was unwilling to finance a departmental manager position because the director of the school, **Johan Larsen**, was "only" qualified as an M.Sc. in Pharmacology. In 1967, on his way home from a renal pathology course for pathoanatomists in Aarhus, Hans Lyon found himself pressed literally against the wall of a compartment in an express train by Professor of Pathology, **Hemming Poulsen**, who was senior physician at the pathology department at Copenhagen City Hospital (PI-KH). Hans promised to manage a histochemical laboratory at PI-KH and to assume responsibility for training within this field at the school for Biomedical Laboratory Scientists.

The first students to complete histo training were already authorised Biomedical Laboratory Scientists and therefore the line-up of players in a world-class histo training at the school for Biomedical Laboratory Scientists in Copenhagen was complete. We had laboratories with histo-trained tutors and an educational institution with histo-enthusiasts. **Anne Palle Andersen** was a passionate leader. She coordinated with the laboratories and was also a tutor (in the period 1966-1989). **Hans Lyon** was an all-round clinical histo expert and tutor (1967-2002). **Poul Prentø**, an expert in his subject, was a tutor "on and off" from 1967-2005 and **K. J. Pedersen** was censor at the main examination until 1996. Veterinary Surgeon **Erik Hasselager**, M.Sc. also taught classes at the school for many years. There was also an excellent team of histo lab instructors: **Jette Aarsø**, **Birgitte Overgård Nielsen** and **Else Krasnik**. Else Krasnik taught histo until 2013 (Figure 5.1.2).

Figure 5.1.2 The players in world-class histo training: From left: **a)** Hans Lyon, Erik Hasselager, Poul Prentø, **b)** Anna Palle Andersen, **c)** Inger Lindebo Holm, **d)** Jette Aarsø, **e)** Birgitte Overgaard Nielsen og **f)** Else Krasnik. (Inger Lindebo Holm)

5.2: Education and postgraduate training in Histochemistry in Denmark

1964 saw the first draft of a histo Biomedical Laboratory Scientist curriculum. 1965 saw the first general training course. In 1968, the curriculum was formalised (1) but it was not until 1 April 1975 that a government white paper was published *Indenrigsministeriets cirkulære om uddannelse af patolog-anatomiske hospitalslaboranter (Home Office white paper on training for pathoanatomical biomedical laboratory scientists)* (2). No radical changes were made to training in histological techniques/histochemistry for 21 years, until, in 1996, a general training course for medical and Biomedical Laboratory Scientists was introduced.

The training of pathoanatomical/histo-cyto Biomedical Laboratory Scientists was located in Copenhagen and *it was probably the best of its kind in Europe - possibly the best in the world.*

The training took three years. There was primary focus on training in the clinic (26 months at the clinic, 10 months in the classroom). Entrance requirements were a grammar school, sixth form college or similar education. Students were recruited to a pathoanatomy department and paid a wage throughout their apprenticeship.

The goal of the training was to equip the Biomedical Laboratory Scientist to perform analytical work in the laboratory from the first day of employment. The white paper (2) described in detail the techniques and analyses the newly-qualified Biomedical Laboratory Scientist had to know and perform.

The white paper also stipulated requirements for consistent updating of the analysis repertoire in line with technological developments and that tutors at the clinic must have undergone both pedagogical and professional postgraduate training. A new white paper appeared in 1980 (3) adding clinical cytology to the curriculum. Over the years, the white paper was implemented to the letter as new analyses and techniques began to be accepted at pathoanatomy departments, e.g. immunohistochemistry, microwave ovens, *in situ* hybridization and xylene-free preparation. Similarly, in response to new pedagogical trends,

project work involving histological/cytological techniques was introduced in 1984.

The training was generally managed by a pathoanatomy education board comprising representatives from the clinics (physician, lab instructor, senior Biomedical Laboratory Scientist), from the school (academic tutors and lab instructors) and from LaH and with the school's pathoanatomy study coordinator/department manager. The study coordinator was responsible for day-to-day course management (administration, finances, pedagogy) at the school and for coordination with the laboratories, which was important.

It was not difficult to substantiate, e.g. to a foreign laboratory, that a Danish histo cyto Biomedical Laboratory Scientist was a useful employee. Whenever they were shown to visitors from European and US institutes, the curriculum and project reports were received with great praise and often also with dismay when they learned that none of the results of the many project experiments were published (Figure 5.2.1)

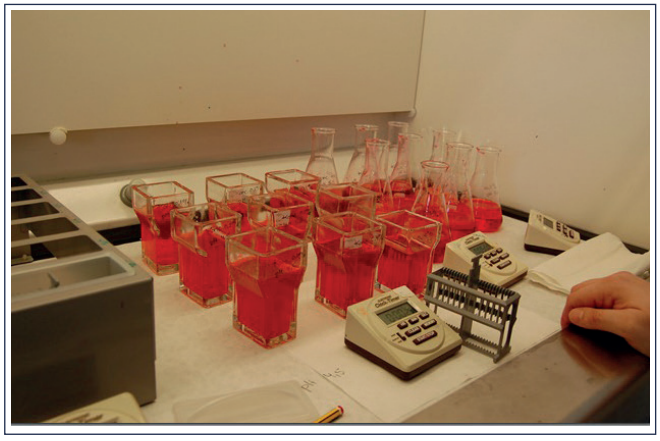


Figure 5.2.1 One of many experiments that were included in the methodologically comprehensive histolaboratory assistant training. Here an examination of the pH-dependent reaction in eosin staining. (Inger Lindebo Holm)

Newly qualified hist-cyto Biomedical Laboratory Scientists were very well trained, not only for practical work but also in assessing and interpreting stainings in order to troubleshoot and control quality. They were also capable of developing methods and disseminating knowledge. The project reports produced in the period 1984-1996 clearly prove this point. If you read the histochemical reports (150-250 pages) box (see the figures: Box 2.4 - Figure 3 and Figure 2.1.7) from the final practical exams, where their level of professional scientific quality and creativity is concerned, they are just as good as today's Bachelor reports. In terms of specific professional scientific expertise, the level achieved by students then was higher than now. Graduates were understandably more adept at analysing results because they had more experience of laboratory routines and a narrower but higher level of professional focus.

5.3: General courses of training for Medical and Biomedical Laboratory Scientists

In 1995 (4), in the wake of a long and tough negotiation process, all the specialist Biomedical Laboratory Scientists training courses were revised. Technological and social developments had made modernising the old white papers imperative and there was a need for more versatile training of medical laboratory assistants in all the medical laboratory specialisms. The three previous study specialisms (clinical chemistry, blood group serology and pathoanatomy) were scrapped. The education was transformed into a general course of training, which trained students to work in all five specialisms (clinical biochemistry, pathoanatomy, blood group serology, microbiology, clinical physiology and nuclear medicine).

From 1996, students were hired via a general application scheme and received a study grant. Until 2001, students were employed at a medical laboratory/unit. During practical work experience periods, the students were attached to a regional or local authority. In addition to the existing institutions in Copenhagen and Aarhus, new educational institutions were opened in Odense, Næstved and Esbjerg and in 2001, all of these became "CVUs" (centres for further education), and in 2008, "Professionshøjskoler" (university colleges). In 2001 (5), the training was subdivided into semesters and became a Bachelor's degree, and, in 2008 (6), it became a modular vocational Bachelor's degree.

Training is no longer targeted at specific practical work functions but aims rather to *qualify the student, on completion of the education, to perform independent bioanalytical work and also to enter into interdisciplinary cooperation*.

5.4: Histochemistry for generalists and biomedical laboratory scientists at professional Bachelor's degree level

In the first general education programme (1996-2001), in addition to cell and tissue preparation, the subject histochemical/histological technique included all the analytical principles performed *in situ* on cells and tissues, i.e. general classical histochemical/staining methods, enzyme histochemistry, immunohistochemistry and cytochemistry and *in situ* hybridization. These skills were taught relatively late in the education programme and new analysis methods were included in the curriculum. The curriculum itself was adjusted and prioritised in accordance with technological development. At the clinic (if this was a pathoanatomy unit), the student received a thorough theoretical and practical training.

Unfortunately, at the time of the transition to general education in 1996, it had become politically incorrect to address analyses specifically according to specialism. When the vocational Bachelor's degree course scheme was abolished in 2001, it was advisable not to state to which specialism an analysis belonged.
This meant that histochemistry was a biomolecular biochemical analysis (KBA) course in the second semester and that enzyme histochemical, immunohisto- and cytochemical and molecular biological histochemical analysis methods were moved to other fields of analysis that were not tissue-related. Enzyme histochemistry disappeared completely and immunohistochemistry (which was an increasingly important part of the profession) was reduced to a two-hour lecture and a single exercise in connection with a problem-based learning (PBL) course in the fourth semester.

Molecular biological histochemistry – also developing by leaps and bounds – was represented in a single exercise in the fourth semester coupled with a clinical cytological PBL course.

In the current modular Bachelor's degree curriculum (two modules each semester), biomolecular chemical biochemical analysis (KBA) is renamed Histochemistry – but not much else has changed: Module 3 includes a review of tissue preparation, a fixation exercise, dye staining theory and a review of HE and PAP stains including practical exercises – totalling 36 hours of study. In Module 6, there is a lecture on and an exercise in connective tissue staining and a lecture on and an exercise in carbohydrate staining. Immunohistochemistry and the molecular biological *in situ* methods are in Modules 8 and 10, each with one lecture and one exercise. A lecture on image assessment and a practical analysis of determining the number of oestrogen receptor-positive cells is added to the immunohistochemical exercise.

Students who elect to do a histochemistry-related Bachelor dissertation at a pathoanatomy unit are offered special training (20 European Credit Transfer System (ECTS) points), corresponding to two-thirds of a semester.
The transition to a general education obviously required many changes and reductions in specialism-related areas of study compared to how much of this was taught in a specialism-specific education. The aim of histochemistry training is now to acquaint students with analysis principles, which apply to many different tissue stains based on the same principle – and on quality control of such analyses. Unfortunately, an understanding of the classical principles of histochemistry presupposes comprehensive knowledge of general, organic and biochemistry and physical chemistry and histology. Like histochemistry, the teaching of these natural science subjects is strictly limited in the present Bachelor's degree course (compared to specialist training), which makes it extremely difficult for the student to play all the strings he or she needs to understand the mechanisms involved even in a haematoxylin-eosin stain on formalin-fixed tissue.

Current status is, however, far from catastrophic. Thanks to the professional expertise of strongly motivated tutors at schools and clinics and, not least, excellent teaching materials, which, in line with reductions in the number of classes taught, have been revised to make them more reader-friendly and visually appealing.

The importance of training at the clinic is immeasurable. Tutors and instructors have undergone specialism-specific postgraduate training (IL) or a diploma. The tutors and therefore the training at the clinics – theory, guidance and practical training – are of an extremely high standard.

5.5: Histochemistry teaching materials

Many highly motivated tutors of histochemistry in Denmark have had the itch to write. The teaching materials available in Danish have always been comparable with

foreign literature. Initially, teaching materials were notes handed out to students, which were then correlated into compendia and books (Figure 5.5.1). Publications include:

- *Det teoretiske grundlag for histologiske og histo-kemiske metoder (The theoretical foundations of histological and histochemical methods)* by Hans Lyon and Poul Prentø, 1969

This compendium was later expanded and published as two text books:

- *Histokemi I og Histokemi II* (Histochemistry I and Histochemistry II) by Hans Lyon, Anne Palle Andersen, Erik Hasselager, Poul Erik Høyer, Morten Møller, Poul Prentø and Bo van Deurs, DSR forlag (printing house), Landbohøjskolen (veterinary and agricultural college) 1985.

- *Theory and Strategy in Histochemistry. A Guide to the Selection and Understanding of Techniques* by Hans Lyon, Anne Palle Andersen, Erik Hasselager, Poul Erik Høyer, Morten Møller, Poul Prentø and Bo van Deurs. Springer Verlag (printing house) 1991.

After the shift to a more general Biomedical Laboratory Scientist training, Histochemistry I and II were too comprehensive and the following compendium was published as a replacement:

- *Kompendium i Kemiske og Biokemiske Analyseprincipper (KBA) ((A Compendium of Chemical and Biochemical Analysis Principles)*
  - *Celle- og vævspræparation (Cell and tissue preparation)*
  - *Vævsfarvning og farvningsmekanismer (Tissue staining and staining mechanisms)*
  - *Farvning af væv på basis af covalent binding og redoxprocesser (Tissue staining based on covalent bonding and redox reactions)* by Hans Lyon and Poul Prentø, 1998, Bioanalytikeruddannelsen (biological laboratory scientist training school).

In the Bachelor's degree, the natural sciences as basis subjects were decimated and in the histochemistry curriculum, the compendium became too difficult. The following has since been used at Metropolitan University College (in Copenhagen):

- *Kompendium i Histokemi (A Histochemistry Compendium)*
  - *Vævspræparation og vævsfarvning (Tissue preparation and tissue staining)*
  - *Bindevævs- og kulhydratfarvninger (Connective tissue and carbohydrate staining techniques)*
  by Inger Holm, Bioanalytikeruddannelsen (biological laboratory scientist training school) 2013.

Via University College uses the following compendium:

- *Noter i farvning af celler og væv, november 2006 (Notes on cell and tissue staining, November 2006)*
  by K. Hallager, V. Jelsbak and T. Meyer.

Both colleges use the following in immunohistochemistry teaching:

- *Anvendt Immunhistokemi (Applied Immunohisto-chemistry)* (current/ 7th edition, 2007), Bioanalytikeruddannelsen (biological laboratory scientist training school) in Copenhagen.
  by Mogens Vyberg,

In addition to books and compendia, teaching materials have normally included other learning aids, e.g. exercise guidelines, assignments and illustrations. Since the introduction of the Bachelor's degree, animations of dye bonding mechanisms and most recently (2013) virtual microscopy with histochemical annotations have helped students to learn complex issues in a short time.

Bioanalytikeruddannelsen (biological laboratory scientist training school) has also published a number of articles in international journals (7-14).

Even though Denmark no longer offers a world-class basic histochemistry education, a student who joins clinical practise at a pathoanatomy department and writes his or her Bachelor project here can be regarded as one of the best histo-trained people around.

5.6: Postgraduate training in Histochemistry

Postgraduate training in histochemistry can be subdivided into two categories: a) the one-year formal postgraduate training course, which trains medical and biomedical lab instructors, for which the minimum entry qualifications are two years of laboratory experience, and b) postgraduate

training available in the form of a range of courses offered by schools, trades unions, professional societies and private course providers.

5.6.1: Histochemistry further education ~ IL courses 1973-1990

The first training course for senior Biomedical Laboratory Scientists and Biomedical Laboratory Scientist tutors was founded in 1960. In addition to leadership and pedagogy, the course had clinical content. In 1973 the first further education course with a specific pathoanatomy module was run. The next course ran in 1976. In 1975, a working committee was formed. In January 1978, it published a report, *Betænkning vedrørende videreuddannelse af hospitalslaboranter (Report regarding further education of Biomedical Laboratory Scientists)*.

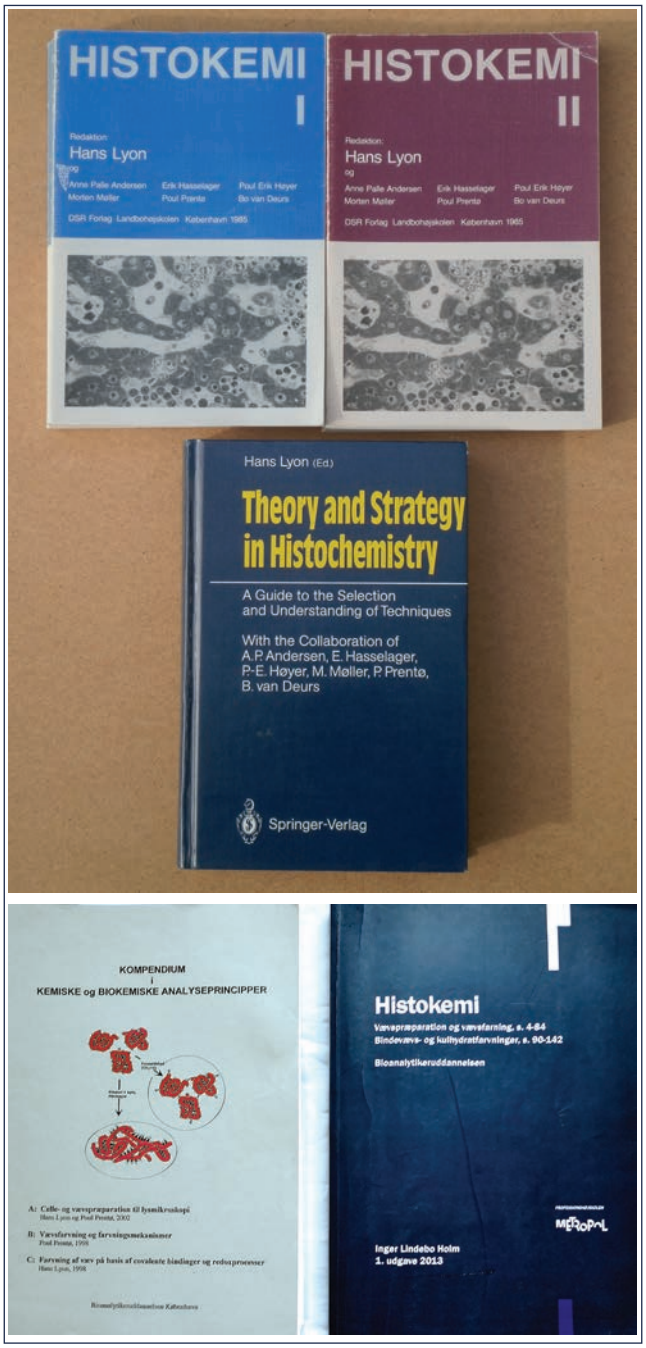


Figure 5.5.1 A selection of Danish histochemistry textbooks

The following were members of a sub-group, which focused on pathoanatomy: Senior Chief Biomedical Laboratory Scientist **Ingeborg Eilertsen**, Biomedical Laboratory Scientist tutor **Ingelise Rohleder**, Professor **Steen Olsen, MD**, Senior Physician **Ulrik Henriques, MD**, School Director **Johan Larsen** and Senior Physician **Hans Lyon**. They proposed a specific pathoanatomy/histochemical (615 teaching sessions) module including natural science support subjects and histochemical/histological techniques (270 teaching sessions). The sub-group envisaged that all teaching would be conducted by academics with teaching experience and as many subject-specific qualifications as possible.

In June 1979, *Indenrigsministeriets cirkulære vedrørende videreuddannelse af hospitalslaboranter (The Home Office circular regarding further education of Biomedical Laboratory Scientists)* (15) was published. The text spelled out the analytical and diagnostic exercises (in addition to theoretical teaching) future Biomedical Laboratory Scientist histochemistry tutors had to undergo. The 20 examples of analytical exercises included eosin structures, ionisation and dye bonding, blocking/unblocking of reactive groups, and immunohistochemistry on correctly and incorrectly fixed tissue. The examples of diagnostic exercises included lipid composition in medulla spinalis, lipid presence in the adrenal gland, presence of hydrolases in the gastrointestinal tract and amino acids in dental structures! The circular left no stone unturned.

Pathoanatomy IL courses were held at Hospitals-laborantskolen (school for Biomedical Laboratory Scientists)in Copenhagen at 3- to 5-year intervals. In 1988, clinical cytology was added to the curriculum and the number of hours of subject-specific pathoanatomy module was increased to 735 teaching hours. At the same time, the teaching was modernised to include workshop teaching, student lectures and an analytical diagnostic project.

5.6.2: Modular further education – IL 1990-1994 (-98)

At the end of the 1980s, the schools in Copenhagen and Aarhus together began to revise further education within the bounds of the circular. Their efforts resulted in a new training course, *Modulopbygget Videreuddannelse til Ledende og Instruerende Hospitalslaborant (Modular further education for senior Biomedical Laboratory Scientists and teachers.* This was a one-year training course comprising four modules: a joint natural sciences (basic subjects) module (300 hours) and a subject-specific further education module in pathoanatomy (students chose between clinical chemistry, blood group serology, clinical microbiology, clinical physiology and nuclear medicine)(400 hours). There was also a leadership module (120 hours) and a pedagogy module (140 hours). The first pathoanatomy/histo module ran in 1993. In addition to classical histochemistry, it included gene technology, epitope chemistry/immunohistochemistry, flow cytometry, confocal laser scanning microscopy, clinical patho diagnostic seminars, themed work and a project.

With effect from 1995, further education was restructured in three modules. The modules were offered alternately in Aarhus and Copenhagen. However, this model was no great success. In the meantime, the Danish Ministry of Education set up a working group, whose mandate was to create a specific diploma course of further education specifically for biomedical laboratory scientists.

5.6.3: Diploma course for biomedical laboratory scientists (HLD) 1998-2001

In September 1997 (16), the Danish Ministry of Education published an executive order, which transformed the modular course of further education (IL) into a one-year Diploma course (HLD). The new course contained the same three modules. However, the general module offered students insight into the analysis methods used in all the specialisms, and the specialist module was optimised. Teaching in histochemistry included tissue staining, quality control, image analysis and screening methods, molecular genetic methods, immunocyto- and histochemistry, microbiology and clinical patho-diagnostic seminars, in all a total of about 200 hours in Module III. 50-60 hours were devoted to a pathoanatomy dissertation. Specific pathoanatomy modules were then held in four consecutive years.

The Diploma course was almost perfect despite the tightly scheduled teaching in specialisms. The professional content was challenging but relevant. As it was possible to hold specialism-specific modules for only 3-5 students (the schools managed finances themselves and the Diploma training did not necessarily have to break even), and even the smallest specialisms succeeded in putting Diploma students through the course every year.

The many excellent tutors of histochemistry involved in further education courses included: **Hans Lyon, Poul Prentø, Per Prætorius Clausen, Mogens Vyberg** and **Ole Nielsen.**

5.6.4: Health Studies Diploma courses (SD) 2002-2011 "Biomedical and Laboratory Technologies" "Professional Practice"

In 2000, the Danish Ministry of Education decided to replace the Diploma course for biological laboratory scientists with a one-year modular Health Studies Diploma course and to integrate all the healthcare professions in the new course. The new Diploma courses appeared at the same time as the CVUs (centres for further education), after which time all postgraduate and further education in the health sector was centralised (EVU) – i.e. separated from the basic training. The executive order on Health Studies Diploma training courses was published in May 2002 (17) and initially resulted in eight different one-year study directions. The study direction that best matched for biological laboratory scientists was entitled "biomedicine and medical laboratory technologies".

Characteristic of the new Health Studies Diploma courses is that, to a great extent, they give students an opportunity to attain much wider competences in relation to biological laboratory work as a profession. It was, however, still possible for students to select a specific module (Specialist Biomedicine: Pathoanatomy, 9 ECTS/6 weeks) and a final project e.g. in histochemistry (15 ECTS/10 weeks). The pathoanatomy module was, however, only offered if there were sufficient students (>12). This happened only once in the period 2002-2011.

In 2011, the eight study directions were reduced to three. "SD i Professionspraksis (Health Studies Diploma in professional practice)" is the study direction that best meets the needs of biomedical laboratory scientists. Specific histochemical content is minimal, although students can

choose modules (corresponding to a total of 20 ECTS/13 weeks) in "Biomedicine and bioanalytical interpretation", "Quality control and development of laboratory analyses" and "A bioanalytical understanding of analyses". Finally, students can choose to write their final project (15 ECTS) on a histochemistry topic.

The current Diploma course does not therefore provide students with an opportunity to gain specific skills in histochemistry, but rather to learn broad-based skills regarding understanding, analysing, interpretation and quality control of bioanalyses.

5.6.5: Non-formal postgraduate training in histochemistry

Over the years relatively few courses were offered in **classical histochemistry**.
As mentioned above, from the late 1960s and until the late 1970s, the University of Copenhagen offered a comprehensive course *Histologisk, Histokemisk Metodik (Histological, Histochemical Methodology* taught by **K.J. Pedersen** and **Poul Prentø**.

Hospitalslaborantskolen (school for Biomedical Laboratory Scientists) in Copenhagen ran only 2-3 courses taught by **Hans Lyon** and **Poul Prentø**, and maybe only 20 people have taken basic histochemistry lessons as "empty chair fillers".

Helge Andersen and his coworkers held a number of courses in histochemistry at the Institute of Medical Anatomy in Copenhagen in the 1970s and 1980s.

Since 2003, dbio has organised and held histochemistry theme days run by tutors of bioanalytics **Inge Marie Bayer**, **Janne Jensen, Tine Meyer** and **Esben Skovsted**.

While working at Kielberg Consult in the period 1999-2012 and subsequently at MSConsult, **Poul Prentø** held three-day and later two-day courses in "Light microscopic preparation and staining methods."

5.6.6: Non-formal postgraduate training in immunohistochemistry

Quite the opposite applies to **immunohistochemistry**, which has been the subject of many courses.
Training course activity was established in the late 1960s in response to the development of the immunofluorescence microscope and the introduction of interference filters as primary filters, plus the detection of antibodies in the blood of patients suffering from bullous disorders and the presence of ABO antigens in premalignant and malignant mucosa disorders (See 3.3). Working with **Agnete Ingild (AI)** from Proteinlaboratoriet, **Jørgen Rygaard (JR), Svend Larsen** and **Allan Wiik**, Dansk Selskab for Patologi (Danish Pathology Society, DPAS) organised annual courses in immunofluorescence techniques.

JR and **Erik Dabelsteen (ED)** were later invited by The American Academy of Oral Pathology to run courses in New Orleans and Kansas City (1974 and 1975), respectively, on the use of immunofluorescence techniques to diagnose disorders of the oral mucosa and in research.

Since the official opening of *The Sino-Danish Biomedical Postgraduate Training Centre,* courses for pathoanatomists were held in 1985, 1987 and 1990. These courses were run by **JR** and **Grete Krag Jacobsen**. The courses included immunohistochemistry classes and laboratory exercises. The tutors were **ED, Gorm Pallesen (GP), Per Prætorius Clausen (PPC), Margit Bæksted (MB)** and **Ole Nielsen (ON).**

From 1986 until 1992, the Danish Pathology Society held postgraduate courses in immunohistochemistry.

In the period from 1979 until 1988, under the auspices of LaH, courses in immunohistochemistry were taught by AI, PPC, **Morten Møller** and **Hans Henrik Nielsen**
From 1991-2012, dbio held courses in method parameters and applied immunohistochemistry taught by **AI, Karl Johan Pluzek, MB, ON, PPC** and **Mogens Vyberg**.

From 1997-2009, Kielberg Consult and later MSConsult held courses in immunocytochemistry and -histochemistry. The many tutors included **Lars-Inge Larsson, Bo van Deurs, MV, Ole William Petersen, Hans Lyon, Claus Koch, Niels Heegaard** and **ON.**

Since 2003, a number of courses have been run under the auspices of NordiQC (see 4.3.4).

As for ***in situ*** **molecular biological** analyses, in the past 25 years there has been an increasing supply of short courses at the schools and under the auspices of dbio.

In the period 2001-2011, the Health faculty at Aarhus University held courses for Ph.D students under the title: *Immunhistokemi, In situ-hybridisering og PCR på histologisk materiale (Immunohistochemistry, in situ hybridization and PCR on histological material).* The courses were organised by **Stephen Hamilton Dutoit**. He also taught the courses and was supplemented by a number of in-house and external tutors, e.g. **Henrik Hager, Vibeke Jensen, Carsten Brandt, Tom Nordfeld** and **ON.**

5.6.7: Histochemistry as part of training to become a specialist physician

With the introduction of a new specialist training in 1970 (18), a theoretical training programme was founded, which comprised a number of subordinate courses, including histochemistry taught as part of the pathoanatomy and histology specialism.

Hans Lyon (HL) and **Poul Prentø** taught from the start. In 1979, they were joined by **Per Prætorius Clausen (PPC)** and varying lab assistants. The theoretical teaching was initially relatively comprehensive. The teaching programme was gradually changed to shift the weight of importance to problem-based learning with practical laboratory teaching at students' workplaces.

Immunohistochemistry was initially taught on the immunopathology course by **Jørgen Rygaard (JR), Erik Dabelsteen (ED)** and **Svend Larsen (SL).**
Later, a course in diagnostic methods was taught. This included teaching in histochemistry and immunohistochemistry. Tutors were **HL, PPC, JR, Gorm Pallesen, Elisabeth Ralfkjær, ED** and **SL.**

Since 1991, histochemistry classes were taught by respectively **HL**, **PPC**, **Thomas Hasselager** and (since 2010) **Henrik Hager**. In the same period, immunohistochemistry was taught by **Mogens Vyberg** and **Ole Nielsen**.

Since 1991, *in situ* hybridization has been taught by respectively **Claus Bøgelund Andersen, Helle Broholm, Mette Klarskov Andersen** and **Birgitte Preiss.**

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CHAPTER 6: THE STORY OF DANISH SOCIETY OF CYTO- AND HISTOCHEMISTRY (DSCH)

by Karina Norring Hjort

6.1: THE EARLY YEARS OF DSCH

The *Danish Society of Cyto- and Histochemistry* (DSCH) was founded in 1976. **Hans Lyon** and **Poul Prentø** took part in a meeting organised by The Royal Microscopical Society in Nottingham, UK on 16-18 September 1975. Among the main topics of this meeting was an informal discussion of the opportunities for European societies to work more closely and to found a European Federation of Histochemists. It was decided to found the society at an upcoming congress in Bucharest in August 1976.

Then, a small group of Danish histochemists, led by **Helge Andersen**, were inspired to investigate whether there was an interest in founding a Danish Histochemistry Society. The group discovered that there was overwhelming interest and about 12 people (representing many specialisms) took part in an inaugural meeting on 9 November 1975.

The first general meeting was held on 30 April 1976, at which the first board was elected. The society's first board comprised:

- Chairman **Poul Erik Høyer**, Assistant Professor, Institute of Medical Anatomy, University of Copenhagen
- Vice-Chairman **Hans Peter Philipsen**, Professor, Department of Odontology - School of Dentistry, Aarhus
- Treasurer **Helge Andersen**, Associate Professor, Institute of Medical Anatomy, University of Copenhagen
- Secretary **Hans Lyon**, Senior Physician, Department of Pathology, Københavns Kommunehospital (still a member of the DSCH board and life member)
- Secretary **Poul Prentø**, Associate Professor, Department of Zoology at The University of Copenhagen
- Accountant **Henning Jensen**, Senior Physician, Deparment of Pathology at Rigshospitalet
- Alternate for Accountant **Johan Larsen**, M.SC. in Pharmacology, Director of School of Medical Laboratory Scientists, Copenhagen

The Danish society's affiliation with *The International Committee for Histochemistry and Cytochemistry* (ICHC) was achieved in August 1976 in Bucharest during the 5th International Congress on Histochemistry and Cytochemistry, which was attended by Helge Andersen, Poul Erik Høyer, Hans Lyon and **Bo van Deurs**.

ICHC was founded in Paris in 1960. Its articles of association were approved at the congress in Frankfurt in 1964. ICHC is an umbrella organisation, which represents societies in many countries across the globe, including: Bulgaria, China, The Czech Republic, Denmark, Finland, Germany, Hungary, Israel, Italy, Japan, The Netherlands, Poland, Romania, The Slovak Republic, Spain, Turkey, UK and USA.

At the congress held in Brighton, UK in 1980, the society changed its name to *The International Federation of Societies for Histochemistry and Cytochemistry* (IFSHC) (See http://ifshc.com). DSCH has always been a member of IFSHC and is always represented at the Congress on Histochemistry and Cytochemistry which is held about once every four years.

The original objective of the society were (1976):

1. to correlate cyto- and histochemical data with biochemical cell and biological tissue data
2. to help develop, define and standardise cytochemical methods with special focus on control analyses and quantification
3. to safeguard the interests of researchers working within cytochemistry and maintain contact with societies in other countries, including participating in international meetings, etc.
4. to simplify exchange of information between Danish research scientists with regard to methods, apparatus, research issues, etc.

Members became members free of charge when they had published at least one article. Others joined as temporary or associate members.

6.2: Updating the objects clause in 2004

In 2004, the Board decided to bring the society's objects clause up to date. The new objects clause was adopted by the general assembly later that year.

The society's object is now, via interdisciplinary efforts between people with a biology/diagnostic background to work, as follows:

1. to disseminate knowledge of reagents and methods used in cyto- and histochemistry in the broadest sense
2. to disseminate knowledge of results achieved via cyto- and histochemical methods in an attempt to help optimise processes and procedures
3. to incorporate cyto- and histochemical results into diagnostic work within biology in the broadest sense
4. We wish to appeal to biomedical laboratory scientists and academics and we seek to ensure that representatives of both groups hold seats on the Board.
5. The society holds about two meetings a year, of which one meeting is, if possible, held on Funen or in Jutland.

The new objects clause is open to a more broad-based, clinical focus and for anyone to become a member, both of the Board and of DSCH. The new DSCH focuses on broad-based representation on the Board to ensure that pathologists, academics/research scientists and biomedical laboratory scientists are represented.

At regular intervals throughout the society's history, we have invited international speakers to bring a breath of fresh air to our meetings.

The chairmen of the society have served for many years. In its history so far, the society has only had three chairmen:

- 1976 **Poul Erik Høyer**
- 1985 **Hans Lyon**
- 2003 **Karina Norring Hjort**

Figure 6.2.1 The three chairmen of Danish Society of Cyto- and Histochemistry (DSCH) and one study coordinator Metropolitan University College, Copenhagen (college for medical laboratory technicians). From left: Karina Hjort, Poul Erik Høyer, Inger Lindebo Holm and Hans Lyon. (Inger Lindebo Holm)

6.3: The new DSCH

DSCH has been a very stable organisation with a stable membership fee, which holds 2-3 meetings a year. Some changes have been introduced in connection with modernising the society.

- DSCH's first website was launched in 2001: www.dsch.dk
- We updated our articles of association 2005 and coordinated them with our new objects clause.
- We have introduced network sessions after all scientific meetings, at which participants are invited, over a glass of wine/soft drink and a sandwich, to join a lively discussion with their peers and exchange experiences
- We have opened up for corporate membership.
- We now have seven corporate members. We have also introduced a set of rules for exhibitors. These measures have helped to create healthy finances. In our 40th jubilee year, DSCH can afford to hold a more sumptuous meeting and publish this book.

There is always a good turn-out at our meetings (60-70 people on average) so we predict a rosy future for DSCH for many years to come. Naturally, we expect to modernise regularly in response to developments in our field and any changes progress may bring.

6.4: Meetings and activities

A list of selected DSCH meetings and courses since DSCH's foundation

1976 Zeiss Micro-Videomat II, a new image analyser

1977 Enzyme histochemical studies of segmentation in the renal tubulus proximalis. Auto-radiographic investigation of sodium/potassium-sensitive ATPase. Glutamate dehydrogenase in histochemical procedure. Studies of lactate dehydrogenase isoenzymes in the aorta

1977 Histochemical investigation of diurnal fluctuations in succinate dehydrogenase activity and endogenous Coenzyme Q concentration in corpus pineale in rats

1978 Methodological observations regarding immunohistochemical studies of immunoglobulins

1978 Indoxyl methods for histochemical investigation of hydrolases

1978 A functional description of the cellular population in the human lymph node

1978 Methods of quantification in enzyme histochemistry

1978 Physical/chemical analysis of various dye samples. Pyronin stains at a standardised dye concentration. Immunohistochemical demonstration of oncofoetal antigens in testis tumours

1979 The cytochemical approach to hormone assay

1979 Histochemistry is a question of localization. An example is the use of radioactive labelled inhibitors. Advantages of cytochemistry

1979 Advanced physical studies of pyronin Y

1980 Formaldehyde-induced fluorescence in carcinoid tumours embedded in hydrophilic plastic media

1980 Do sample sections have to be xylene-dehydrated before mounting?

1982 Quantitative cytochemical studies of the metabolism of synovial lining cells with special reference to alterations in rheumatoid arthritis

1983 Fibronectin – immunohistochemical effect and presence in normal and inflamed tissue

1983 Determination of oestrogen and progesterone receptors in mamma cancers

1984 Ultrastructural localisation of beta-galactosidase activity in peritoneal macrophages from C57B1 mouse. A quantitative cytochemical method to measure beta-galactosidase in cultivated human fibroblasts. A microfluorometric method to determine the activity of alpha-galactosidase in cultivated fibroblasts and amniotic fluid cells from Fabry and normal patients

1984 Recent developments in DNA cytochemistry, with particular reference to *in situ* hybridization with non-radioactive labels

1985 Autoradiographic demonstration of receptors for vasoactive intestinal polypeptide (VIP) in corpus pineale. Amygdala kindling of benzodiazepine receptors. Receptor bonding measured using computer-assisted autoradiography

1986 A historic survey of the development of the Romanowsky-Giemsa method. The use of Romanowsky-Giemsa method in veterinary

practice, advantages and problems. The use of the Romanowsky-Giemsa method for staining fine-needle aspiration biopsies, advantages and problems. Problems with the Giemsa stain. The Romanowsky-Giemsa stain, Standardization and reproducibility as a cytological and histological stain

1987 Stereology. An overview, opportunities, examples and perspectives

1987 Lectins, an overview, opportunities, examples and perspectives

1988 Training course in microwave oven techniques (2 days)

1988 In situ hybridization

1990 Organic solvents in histology

1990 Autometallographic silver enhancement – a histochemical technique to detect gold, silver, mercury and zinc

1991 Workshop in applied immunohistochemistry Methodological issues in immunohistochemistry (2 courses in January and August)

1991 Mini symposium and presentation of The Bartholin Institute. Diabetes research, immunology, and cancer and neurology research

1991 The terminal complement complex - friend or foe?

1992 Quality control in histopathology – Useful or only bothersome?

1993 Workshop in applied immunohistochemistry. Methodological issues in immunohistochemistry

1993 Image analysis

1993 Xylene substitution in the clearing deparaffinization processes

1993 Immunohistochemistry workshop

1994 Enzyme histochemistry in diagnostic pathology. Contemporary approaches

1994 Practical experience of the use of the microwave oven at pathology departments

1994 Current projects from the recent course for senior medical laboratory assistants and lab instructors

1995 Quality control in pathological anatomy. Intentions and experiences from Sweden and Denmark

1995 Opportunistic infections: A joint task for clinicians, microbiologists and pathologists

1995 Estisol 220 – the story so far

1995 PAPNET – A diagnostic or quality management system?

1995 Quantitation in immunohistochemistry

1995 Workshop – Quantitation in immunohistochemistry

1996 A overview of the uses of gene technology

1996 Formalin-free fixation agents. Can they produce good results?

1996 Epitope retrieval

1997 Neuronal regulation of melatonin from the pineal gland

1997 Immunostainers – the pros and cons

1997 Cyclins – Diagnostic and prognostic relevance

1997 Double immunoenzyme staining techniques – an overview

1997 *In situ* hybridization: Quantitative aspects

1997 CYTO97 in York. Margit Bæksted, Hans Lyon and Erik Hasselager took part

1998 PNA – Peptide Nucleic Acid

1998 We do have controls, but do we have quality control?

1999 Immunohistochemical visualisation of receptors

1999 New techniques in diagnostic cervix cytology: thin-layer/monolayer preparation and automated screening

2000 Quality control of IHC – joint meeting with DSP and DSPAC

2000 Training course in PCR Techniques

2000 Immunohistochemical quality control and standardisation

2000 The role of telomeres and telomerase in cancer ageing

2001 Confocal microscopy

2001 Gene Display Systems

2001 Phase Two of gene technology: New techniques – new ways of thinking

2002 DNA analysis with flow and image cytometry – joint meeting with DSFCM

2003 HPV p16

2003 Molecular biology and molecular biological techniques (5-day course)

2003 Mammacancer. Selected prognostic and predictive markers and suggested new therapy methods

2004 New Tools in the Diagnosis of Lymphomas and Leukemias
2004 GIST (gastrointestinal stromal tumour), CML og imatinib: definitions, diagnoses and therapies
2004 Quality Control – joint meeting with NordiQC
2004 News from the past
2005 Angiogenesis and endothelial cell biology
2006 Nordic IHC Quality Control – joint meeting with NordiQC
2006 Molecular techniques
2007 Stem cell research
2007 Advanced fluorescence imaging techniques
2008 Nordic mamma carcinoma: IHC Quality Control – joint meeting with NordiQC
2008 Working and studying abroad
2008 Computer-based quantitative microscopy – with focus on tumour markers
2009 Bridging Oncology and Pathology - joint meeting with DBCG with guest lecturer Prof. Giuseppe Viale, Milan, Italy
2010 Colon cancer: Pathoanatomical research – diagnostic, prognostic and therapeutic markers
2011 Best Laboratory Practice and Standardization of IHC Testing – joint meeting with NordiQC
2012 Pathoanatomical studies and treatment of malignant melanomas
2013 1st Nordic Conference on Standardization in Applied IHC
2013 HER receptors and their role in cancer
2014 Targeted lung cancer therapies – pathology meets oncology
2015 Prostate cancer. Diagnostics, challenges and new initiatives
2015 Gene profile tests – are they ready for the clinic?
2016 HPV Disease and prevention: Screening and vaccine

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