Methods in Neuroendocrine Histopathology
A Methodological Overview

Lars Grimelius

Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden

Abstract
Light microscopy is still the main tool in diagnostic histopathology, though it does not always lead to a definitive diagnosis. It has therefore been a constant ambition to develop methods which can add further information to the diagnosis.

In endocrine pathology, a major problem has been to distinguish between neuroendocrine and non-neuroendocrine tumours. The silver stains, such as the Bodian, Grimelius and Sevier-Munger methods, were the first useful “general neuroendocrine” markers. Electron microscopy can also be useful for identifying neuroendocrine tumours.

A further step forward was the introduction of histochemical fluorescence methods, as these could identify biogenic amines. With the introduction of immunohistochemical techniques, tumours could be characterized in a more specific way regarding peptide hormones and biogenic amines content, proliferation factors, hormone receptors, etc. Another method, DNA cytometry, has been used mainly in predicting the prognosis. In situ hybridization can be a useful complement to the histopathological diagnosis when other methods have failed to demonstrate the neuroendocrine nature of the tumour. Some endocrine tumours, especially the well-differentiated ones, still cause diagnostic problems in predicting tumour behaviour, why further complementary methods would be of great value.

Introduction
Histopathological tumour diagnosis is still based mainly on light-microscopical structure patterns. During the second half of the 19th century, up to the late 1960s several histotechnical stains were developed. In histology and histopathology they became some help in identifying particular cell types, cell structures, and different types of neoplasms. Substances such as lipids, different types of carbohydrates (e.g. glycogen, mucus) could be demonstrated as well as pigment, heavy metals, calcium deposits and amyloid structures, etc. Some of them were introduced in histopathological diagnostic laboratories all over the world. Most of them were developed empirically, based on simple tinctorial reactions, while their precise chemical background remained mainly unknown (1).

The purpose of the present overview is to describe some of the tinctorial and histochemical staining methods used in neuroendocrine pathology during the 20th century and to evaluate their usefulness when compared with that of modern diagnostic procedures.
Silver stains

Silver stain methods have gained a wide field of applications in histology and histopathology (1). Several tissues and cell types can be identified with silver stains, including most types of neuroendocrine cells. Up to the late 1960s, the silver stains mostly used for endocrine cells and tumours were modifications of the Masson, Gros-Schultze and Bodian methods.

In 1914 Masson described a silver stain, initially developed for demonstrating neuronal structures. However, it was soon found that this stain could also visualize Kulchitsky cells in the crypts of Lieberkühn and carcinoid tumours in the small intestinal tract, a tumour type first described by Oberndorfer in 1907 (2–5). These silver-positive intestinal cells are called enterochromaffin (EC) cells. The recognition of carcinoids as neuroendocrine neoplasms was outlined by Gosset and Masson in 1914 (3).

The Masson method, empirically developed, was later modified (6–8), which improved its reproducibility (Fig. 1). This staining method, based on an ammoniacal silver solution, is a “one-step” procedure, i.e. the cells contain chemical substance(s) that can retain silver ions and reduce them to metallic silver without assistance from an external reducer; the cells visualized by this technique were termed “argentaffin”. Baker and Pearse suggested that these argentaffin cells contain serotonin (5-hydroxytryptamine) and that the reaction product of this monoamine with formaldehyde, beta-carboline, causes the argentaffin reaction (9, 10). By using a histochemical model, the dot blot technique, Lundqvist et al. showed in 1990 that also other biogenic amines, such as Dopa, dopamine, norepinephrine, epinephrine and 5-hydroxytryptophan, can give rise to the argentaffin reaction (11).

Another principally different type of silver staining technique was also developed on an empirical basis with an initial silver impregnation of the sections fol-

Figure 1. Midgut carcinoid tumour showing argentaffin reaction (Masson method).
followed by a reduction process by an external reducer. The neuroendocrine cells visualized by this silver technique were called "argyrophil". By using various silver salts, solvent solutions, and reducing substances, different cell types and tissue components could be visualized. Most of these silver staining techniques were initially developed with focus on neural tissue but some were found to be useful also for demonstrating neuroendocrine cells and their neoplasms. One of these stains, the Gros-Schultze technique, later modified by Hamperl, demonstrated argyrophil cells in the mucosa of the gastrointestinal (GI) tract and in the islets of Langerhans (12). However, this method presented a number of technical difficulties and did not give reproducible staining results (13).

Another silver stain, the Bodian method, used a silver proteinate ("protargol") solution for the initial impregnation of the sections, followed by reduction by an external reducer of hydroquinone and sodium sulphite (14). This stain was also initially developed for nerve structures but subsequently modified to demonstrate neuroendocrine cells in mucosa of the GI tract and endocrine pancreas (15, 16). The technique is simple, giving reproducible results, but it was found that only a few of the manufactured batches of the silver proteinate were useful.

Already in 1929/30, Davenport described an alcoholic silver nitrate stain for neuronal structures (17, 18). In Uppsala, Hellerström and Hellman, who modified this staining method in 1960, were able to demonstrate a minor fraction of non-insulin cells in rat pancreatic islets and this cell fraction was termed A1 (19). These A1 cells corresponded to an earlier described cell type in man and other mammals, called D cells (Fig. 2). Polak et al. showed in 1975, partly by a restaining technique, that these A1 cells were actually somatostatin cells, a type also occurring in the mucosa of the GI tract (20).

In 1968 an argyrophil staining method was developed in our laboratory in Uppsala, based on a low silver nitrate concentration at slightly acid pH (21). The reducer was the same as that used in the Bodian stain. The stain was modified slightly in 1980 (22). By using "dot blot" technique, Rindi et al. (23) showed that chrom-

---

Figure 2. Human pancreatic islet stained with the Hellerström-Hellman modification of the Davenport technique. The black cells represent A1 (D) cells.
ogranin A gives rise to the argyrophil reaction and Lundqvist et al. (11) showed that dopamine, norepinephrine and 5-hydroxytryptamine also elicited the argyrophil reaction. The argyrophil cells visualized by the Grimelius method were found to constitute a separate cell type in the islets of Langerhans, different from the A1 and B cells in man (21).

With the introduction of immunohistochemical (IHC) methods, the Grimelius silver-positive cells could be related to specific peptide hormones and biogenic amines. They were found to correspond to the glucagon and pancreatic polypeptide (PP) cells of endocrine pancreas, but also to most cell types in the mucosa of the GI tract (Fig. 3); exceptions were insulin, somatostatin, CCK and polypeptide YY cells (22, 24). An argyrophil reaction was observed in the chromaffin cells of adrenal medulla, but not in the cortex (25). Parenchymal cells of the anterior lobe of the pituitary gland (26), and C cells of the thyroid gland were also argyrophilic, but follicular cells were not (25, 27). Argyrophil cells also appeared in parathyroid gland as well as in other organs (28–30). The neoplastic cells of neuroendocrine tumours derived from these argyrophil cells also demonstrated the silver reaction (Fig. 4). With few exceptions the Grimelius method stained almost all neuroendocrine tumours and was therefore used as a general neuroendocrine marker. A few neuroendocrine tumours (some insulinomas, some hindgut carcinoids and somatostatinomas) lacked the argyrophil reaction (22, 31).

In 1965 Sevier and Munger (32) developed another silver stain for identifying nerve structures, but this stain also visualized endocrine cells in the gastric mucosa (22, 25, 33–36) in the adrenal medulla and C cells of the thyroid gland (37).

In 1979, Churukian and Schenk modified another silver stain, originally developed by Pascual; the modified method is similar to the Grimelius stain; the impregnation solution contains a higher concentration of silver nitrate and its pH is

**Figure 3.** Human pancreatic islet stained with the Grimelius method. The silver–positive cells represent glucagon and PP cells.
lower. The method visualizes endocrine cells in the pancreatic islets and in various neuroendocrine tumours (38).

Electron microscopical analyses of silver-stained sections showed that the metallic silver grains were located in the secretory granules in the Masson, Davenport and Grimelius stains (36, 39, 40). The frequency of silver grains varied within these granules and their localization differed to some extent in the different endocrine cell types (36, 41) (Fig. 5).

The above-mentioned silver stains used in neuroendocrine histopathology diagnosis do not give identical results. The Grimelius, Bodian, Sevier-Munger and Churukian & Schenk methods seem to visualize more or less the same endocrine

*Figure 4.* Electron micrograph of human pancreatic glucagon (A) cell stained with the Grimelius technique. The silver grains are concentrated over the peripheral less electron-dense mantle of the secretory granules.

*Figure 5.* Medullary thyroid cancer stained with the Grimelius technique. A stronger silver reaction is seen in the neoplastic cells facing the fibrovascular stroma. The staining intensity reflects the frequency of secretory granules.
cell populations (22) whereas the Masson and Davenport methods visualize specific cell types. Therefore, when argentaffin and/or argyrophil neuroendocrine cells are described, it is important to clarify the staining method used.

A silver stain has also been used to identify so-called nucleolar organizer regions (NORs) which are believed to consist of proteins associated with ribosomal RNA activity (42), protein synthesis (43), and cell proliferation (44). The stain has been widely applied in a variety of cell kinetic studies, using the mean number of argyrophil “(Ag) NORs” of the tumour cells mainly as a marker for cell proliferation. The staining method, initially developed by Goodpasture & Bloom (42), modified by Howell & Hsu (45) and Li et al. (46), has provided additional information to predict malignancy in some endocrine tumours (47–49).

Non-silver stains

In the pre-IHC era, some neuroendocrine cell types could be visualized by stains other than silver-based. Insulin cells could be demonstrated with Gomori aldehyde fuchsin and chrome haematoxylin stains (50, 51) and also by the pseudo-isocyanin stain after permanganate oxidation (52). Basic dyes, such as toluidine blue and Azure A, were also used to identify endocrine cell types in endocrine pancreas and in the mucosa of the GI tract, in pituitary gland (anterior lobe), and C-cells of the thyroid and adrenal medulla. In addition, diazonium salts and xanthydrol demonstrated neuroendocrine cells. The bromine-alcian blue-OFG method was often used to identify acidophilic, basophilic and chromophobic cells of the pituitary gland and its tumours (53). The lead-haematoxylin method developed by MacConaill (54) and modified by Solcia et al. (25) was also used to also demonstrate neuroendocrine cells in various organs.

Fluorescence histochemical methods

Falck and Hillarp and their co-workers introduced in 1961 a gas phase fluorescence histochemical procedure to demonstrate biogenic amines in neuroendocrine cells (55, 56). This procedure was more sensitive than those previously used. The main reason was that the conventional techniques used at that time involved a water phase, in which inadvertent extraction of biogenic amines in the tissue could not be entirely avoided. The new technique was based on a condensation reaction between gaseous formaldehyde and the biogenic amines in freeze-dried tissues; some specific residues of peptides could also be demonstrated. By exposing the sections to different light excitation spectra, biogenic amines as well as some peptides could be identified (57).

When catecholamines and 5-hydroxytryptamine react with gaseous formaldehyde, the reaction products give rise to a green fluorescence with the aforementioned amines, yellow with the latter (55–57) (Fig. 6). Thus, the adrenal medullary
cells and the EC cells in the mucosa of the GI tract could be reliably visualized by this method. Some cells of the pituitary anterior lobe were also found to display this specific fluorescence (58).

The paraformaldehyde-ozone method is similar to the above-mentioned technique but the condensation reaction takes place in the presence of ozone which acts as an oxidizing catalyst. In histochemical models, tryptamine and peptides with the NH2-terminal tryptophan give rise to a yellow fluorescence (59, 60). With this method, ACTH, gastrin and glucagon cells as well as C-cells of the thyroid display fluorescence (61–64).

If the paraformaldehyde condensation process takes place in an acid environment (the formaldehyde-HCl method), intense fluorescence is seen in a variety of cells, not only in the neuroendocrine. Tryptophan residues, irrespective of their position in the peptide molecule, were found to give rise to fluorescence. Gastrin and other cells in the mucosa of the GI tract, as well as the pancreatic glucagon cells, were demonstrated by this method (65, 66).

Histochemical models have shown that the reaction products between o-phthalaldehyde and histamine, as well as NH2-terminal histidine, give rise to fluorescence (67). This aldehyde probably reacts with other proteins too. Mast cells and EC-like cells are visualized by this method, as glucagon cells. All three cell types are equipped with peptide hormones or biogenic amines, containing histidine in the NH2-terminal position (67–72).

The fluorescamine method is another technique, that in formaldehyde-fixed tissue specimens can be used to demonstrate certain peptide hormone-secreting cells such as pituitary GH cells, gastrin and insulin cells, as well as the C-cells of the

Figure 6. Unstained antral mucosa section fixed with paraformaldehyde showing enterochromaffin cells. The reaction product between paraformaldehyde and 5-hydroxytryptamine, beta-carboline, gives rise to the yellow fluorescence.
However, it was found that this fluorescence was not related to the presence of the respective hormones but rather to some granule component(s) distinct from the hormones (73–76).

**Immunohistochemistry**

The immunofluorescence technique was introduced in 1941 by Coons et al. (77). Initially, a direct staining procedure was used, *i.e.* the primary antibodies were la-
Methods in neuroendocrine histopathology

belled with a fluorophore. During the 1950s, with the introduction of the unlabelled antibody (sandwich) method, the staining procedure became simpler, more sensitive and useful for the characterization of neuroendocrine cells and tumours (78). The primary antibody was unlabelled, but the second, which was raised against the immunoglobulins of the species from which the primary antibody was raised, was labelled. Initially the antibodies were polyclonal, i.e. raised against several epitopes. By raising antibodies to a limited amino acid sequence, their specificity was increased and the introduction of monoclonal antibodies also improved their specificity. The staining sensitivity was further increased by the introduction of the PAP (peroxidase-antiperoxidase) (79, 80), ABC (avidin-biotin complex) (81), and biotin-streptavidin methods (82, 83). Further improvements in sensitivity were achieved with tyramide signal amplification techniques and polymer-based detection systems (84, 85).

Double- and even triple-immunostaining can be performed in order to identify co-localization of two and three proteins, respectively, in the same cells in the same section (86) (Fig. 7 A–D). With this staining technique, fluorophores are preferred to chromogens, as it is possible to alternate between single and double/triple stainings by changing the filter sets. In single immunostaining, a variety of chromogens can be used, of which diaminobenzidine and amino-ethyl carbazole are the most common.

The secondary antibody can be labelled with gold particles of nano size and the protein in question can then be identified in the electron microscope (Fig. 8). An ultrastructural co-localization of different proteins can be performed by labelling the various antibodies with gold particles of different size (87).

Holgate et al. (88) introduced the immunogold-silver stain for light microscopy. The method was modified by Hacker et al. (89, 90) (Fig. 9). The secondary antibodies were labelled with gold particles of nanometer size, but these particles are too small to be identifiable under the light microscope. By applying a silver acetate autometallography method, the gold particles catalysed the reduction of silver ions to

Figure 8. Electron microscopic picture of an enterochromaffin cell from rectum, immunostained with serotonin antibodies labelled with colloidal gold particles (15 nm). The gold particles are concentrated to the secretory granules.
metallic silver on the surface of the gold particles. These gold-silver particles grow in size until they become visible even under the light microscope (91).

In diagnostic histopathology, formalin is the main fixative. The fixation process induces various reactions between the fixative and tissue proteins, often with the formation of “cross-links” between protein end-groups, which may mask epitopes. However, these cross-links can be disrupted by using antigen retrieval techniques (exposure to high temperature, enzymatic, or strong alkaline treatment) which unmask the epitopes and increase the usefulness of IHC for routine histopathological diagnosis (92).

IHC techniques have become an integral part of histopathological diagnosis, particularly for tumour characterization. The IHC staining process is now largely performed, to a great extent, by using automated devices.

DNA cytometry

Cytometrical assessment of the nuclear DNA content of tumour cell nuclei was introduced as an additional tool in attempts to conduct histopathological grading of the malignancy of the neoplasms and in efforts to evaluate their prognosis, supplementary to the findings of conventional clinical and histopathological investigations.

In 1924 Feulgen and Rossenbeck (93) described a fluorescence staining method for DNA, and showed that the intensity of the fluorescence could be assessed by a microspectrophotometric technique. Caspersson and colleagues developed a high-resolution scanning technique which allowed measurement of DNA by the fluores-
cence intensity of each individual cell (94, 95). These innovations formed the basis for the development of image DNA cytometry. Nowadays, this kind of analyses can be performed not only on imprints or fine-needle aspirates but also on thin, deparaffinized histopathological sections, again using a cytophotometric method based on light-transmission measurements of "Feulgen-stained" nuclei. With the introduction of flow DNA cytometry, a greater number of nuclei could be analysed (96) but the results were invalidated by the fact that in flow DNA cytometry one cannot be certain that the nuclei analysed are neoplastic cells. However, the cytommetric DNA assessment is of mainly prognostic value in neuroendocrine pathology (97–100).

Electron microscopy

When a pathologist is in doubt whether or not a neoplasm is of neuroendocrine nature, the ultimate recourse is electron microscopy, the reason being that neuroendocrine cells, by definition, contain secretory granules. Their size, density and configuration can differ somewhat between the different cell types. These differences are, however, not retained in neuroendocrine tumour cells, with some exceptions, i.e. the crystalline configuration of the granules seen in normal insulin cells can often be recognized in insulinomas. Ultrastructural examinations are thus used chiefly to distinguish between neuroendocrine tumours and other tumour types (101).

**In situ** hybridization

Neuroendocrine tumours can be functioning or non-functioning. The former tumour type gives rise to symptoms of hormonal overproduction, the latter type not. Sometimes it can be difficult to ascertain the hormone in question by immunohistochemical means, either because (i) the epitopes have become masked, or (ii) the amount of stored protein is below the detection limit, or (iii) the hormone has been released, or (iv) is not synthesized. In such a situation **in situ** hybridization can help. This technique can allow one to demonstrate the presence of mRNA sequences specific for the peptide hormone or the biogenic amine in question. Probes labelled with isotopes, chromophores ("CISH"), or fluorophores ("FISH") are used in histopathological diagnosis (102–106). The sensitivity can be improved as in conventional IHC methods, e.g. by tyramide amplification (84, 107).

Another sensitive non-fluorescence method in **in situ** hybridization is the use of probes labelled with colloidal gold of nanometer size. By using the same amplification process as for the IHC immunogold-silver technique, the gold-silver particles grow in size until visible in the light microscope (108–110).
Density gradient technique

A common issue in daily routine histopathological diagnostic service is to assess the structural basis for the symptoms of a patient suffering from hyperparathyroidism. Here the parathyroid histopathological diagnosis is based on glandular weight, cellular appearance and arrangement and also clinical information. Normal parathyroid glands in adults consist of parenchyma and interstitial fat cells. The quantity of fat cells varies to some extent with age and body constitution. Thus obese persons usually have a greater fat cell content than lean persons. Even pathological glands can contain fat cells, especially hyperplastic glands. The distribution pattern of fat cells can thus vary to some extent in both normal and abnormal glands. In the histopathological diagnosis, the parenchymal cell mass plays an important role as it reflects the gland’s functional state. Parenchymal cell weight can be calculated easily by using a percoll density gradient, a rapid and simple method, also useful in intraoperative diagnosis (111–112).

General conclusions

With the introduction of the Grimelius silver stain it was possible in an easy way to distinguish between neuroendocrine and non-neuroendocrine tumours. With the introduction of IHC techniques, neuroendocrine tumours can be carefully analysed regarding their peptide hormone and biogenic amine content, growth factors, hormone receptors, etc. The IHC tumour cell proliferation index is a useful complement to the conventional assessment of mitotic activity in trials to establish a prognosis.

Electron microscopy, in situ hybridization, and density gradient technique can also serve as complementary methods for the characterization of neuroendocrine tumours.

The traditional “old” signs of histopathological assessment of malignancy, such as the occurrence of metastases, infiltrative growth into the peritumoral tissue, and the invasion of blood and lymphatic vessels, are still the most useful signs for confirming histopathological malignancy. However, the well-differentiated and the more or less well demarcated neuroendocrine tumours still often cause diagnostic problems. Tumour size and tumour cell proliferation can be of importance to predict tumour behaviour, but the appearance of necroses and atypical mitoses can also be of important diagnostic help. For these well-differentiated tumour groups, there is a need for further diagnostic tumour markers.

Acknowledgements

I am grateful for valuable comments on this article from the present and previous collaborators: Professors Sture Falkmer, Guida M Portela-Gomes, Henry Johans-
son and Gerhard W Hacker. I also thank Professor Guida Portela-Gomes and Agneta Lukinius, PhD for contributing with Figs. 6 and 8, respectively and Apostolos Tsolakis MD, PhD for arranging the figures.

This work has been supported by the Swedish Medical Research Council and the Lions Foundation.

References
1. Romeis B (1968) Mikroskopische Technik. R Oldenbourg Verlag, Wien.
2. Masson P (1914) La grande endocrine de l’intestin chez l’homme. CR Acad Sci (Paris) 138:59–61.
3. Gosset A, Masson P (1914) Tumeurs endocrines de l’appendice. Presse Med 25:237–240.
4. Masson P (1924) Appendice neurogène et carcinoides. Ann Anat Pathol Med-Chir 1:3–59.
5. Obersdorfer S (1907) Karzinoides Tumoren des Dünnarms. Frankf Z Pathol 1:426–432.
6. Hamperl H (1927) Über die "gelben" (chromaffine) Zellen im gesunden und kranken Magendarmsschlauch. Virchows Arch 321:482–548.
7. Singh I (1964) A modification of the Masson-Hamperl method for staining of argentaffin cells. Anat Anz 115: 81–82.
8. Portela-Gomes GM, Grimelius L (1986) Identification and characterization of enterochromaffin cells with different staining techniques. Acta Histochem 79:161.
9. Barter R, Pearse AGE (1955) Detection of 5-hydroxytryptamine in mammalian enterochromaffin cells. Nature 172:810.
10. Barter R, Pearse AGE (1955) Mammalian enterochromaffin cells as the source of serotonin (5-hydroxytryptamine). J Pathol Bacteriol 69:25–31.
11. Lundqvist M, Arnberg H, Candell J, Malmgren M, Wilander E, Grimelius L, Öberg K (1990) Silver stains for identification of neuroendocrine cells. A study of the chemical background. Histochem J 22:615–623.
12. Hamperl H (1932) Was sind argentaffine Zellen? Virchows Arch 286: 811–833.
13. Creutzfeldt W (1953) Zur Deutung des Silberzellbildes und anderer Pankreasbefunde beim Diabetes mellitus und Insuladenom. Beitr Pathol Anat 113:133–168.
14. Bodian D (1936) A new method for staining nerve fibres and nerve endings in mounted paraffin sections. Anat Rec 65:89–97.
15. Hamperl H (1952) Über argyrophile Zellen. Virchows Arch 321:482–507.
16. Grimelius L (1964) A modified silver proteinate method for studying the argyrophil cells of the islets of Langerhans. In: Brolin SE, Hellman B, Knutson H (eds); The Structure and Metabolism of the Pancreatic Islets. Pergamon Press, Oxford; pp 99–104.
17. Davenport HA (1930) Staining nerve fibers in mounted sections with alcoholic silver nitrate solution. Arch Neurol Psykiat 24: 690–695.
18. Davenport HA (1929) Silver impregnation of nerve fibers in celloidin sections. Anat Rec 44:79–83.
19. Hellerström C, Hellman B (1960) Some aspects of silver impregnation of the islets of Langerhans in the rat. Acta Endocrinol (Copen) 35: 518–532.
20. Polak JM, Pearse AGE, Grimelius L, Bloom SR, Arimura A (1975) Growth-hormone release-inhibiting hormone in gastrointestinal and pancreatic D cells. Lancet 1:1220–1226.
21. Grimelius L (1968) A silver nitrate stain for A2 cells in human pancreatic islets. Acta Soc Med Upsal 73: 243–270.
22. Grimelius L, Wilander E (1980) Silver stains in the study of endocrine cells of the gut and pancreas. Invest Cell Pathol 3: 3–12.
23. Rindi G, Buffa R, Sessa F, Tortora O, Solcia E (1986) Chromogranin A, B and C immuno-reactivities of mammalian endocrine cells. Distribution, distinction from co-stored hormones/prohormones and relationship with argyrophil component of secretory granules. Histochemistry 85: 19–28.
24. El-Salhy M, Grimelius L, Wilander E, Ryberg B, Tenerius L, Lundberg JM, Tatemoto K (1983)
Immunocytological identification of polypeptide YY (PYY) cells in the human gastrointestinal tract. Histochemistry 77:15–23.

25. Solcia E, Capella C, Vassallo G (1969) Lead-haematoxylin as a stain for endocrine cells. Significance of staining and comparison with other selective methods. Histochemie 20: 116–126.

26. Urbanski SJ, Kovač K, McComb DJ, Ryan N (1982) Argyrophil granules in the human pituitary. Acta Histochem 70: 69–77.

27. De Grandi P (1970) The routine demonstration of C cells in human and animal thyroid glands. Value of a simple silver stain. Virchows Arch B Cell Pathol 6:137–150.

28. Grimelius L, Johansson H, Lindquist B, Wibell L (1972) Tertiary hyperparathyroidism occurring during a renal transplantation programme. J Pathol 108:23–33.

29. Frigerio B, Capella C, Wilander E, Grimelius L (1982) Argyrophil reaction in parathyroid glands. Acta Pathol Microbiol Scand [A] 90:323–326.

30. Capella C, Solcia E (1971) Optical and electron microscopical study of granules in human carotid body, carotid body tumours and glomus jugulare tumours. Virchows Arch B Cell Pathol 7:37–53.

31. Creutzfeldt W (1975) Pancreatic endocrine tumors – The riddle of their origin and hormone secretion. Israel J Med Sci 11: 762–776.

32. Siever AC, Munger BL (1965) A silver method for paraffin sections of neural tissue. J Neuropathol Exp Neurol 24: 130–135.

33. Grimelius L (2003) Silver stains demonstrating neuroendocrine cells. Biotechnic Histochem 79:37–44.

34. Black WC, Haffner HE (1968) Diffuse hyperplasia of gastric argyrophil cells and multiple carcinoid tumors. Cancer 21:1080–1099.

35. Solcia E, Capella C, Vassallo G (1970) Endocrine cells of the stomach and pancreas in states of gastric hypersecretion. Rendic R Gastroenterol 2:147–158.

36. Vassallo G, Capella C, Solcia E (1971) Endocrine cells of the human gastric mucosa. Z Zellforsch Mikros Anat 118:49–67.

37. Wilander E, Juntti-Berggren L, Lundqvist M, Grimelius L (1980) Staining of rat thyroid parafollicular (C-) cells with the Sevier-Munger technique. Acta Pathol Microbiol Scand [A] 88: 339–340.

38. Churukian CJ, Shenk EA (1979) Modification of Pascual`s argyrophil method. J Histotechnology 2: 102–103.

39. Grimelius L, Strand A (1974) Ultrastructural studies of the argyrophil reaction in A1 cells in human pancreatic islets. Virchows Arch A Path Anat Histol 364: 129–135.

40. Grimelius L (1969) An electron microscopic study of silver stained adult human pancreatic islet cells, with references to a new silver nitrate procedure Acta Soc Med Upsal 74:74: 28–48.

41. Solcia E, Polak JM, Pearse AGE, Forssmann WG, Larsson L-I, Sundler F, Lechago J, Grimelius L, Fujita T, Creutzfeldt W, Gepts W, Lefranc G, Heitz PH, Hage E, Buchan AMJ, Bloom SR, Grossman MI (1978) Lausanne 1977 classification of gastroenteropancreatic endocrine cells. In: Bloom SR (ed); Gut Hormones. Churchill Livingstone, London; pp 40–48.

42. Goodpasture C, Bloom SE (1975) Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma (Berl) 53:37–50.

43. Plosten D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ (1986) Improvement in the staining and the visualization of the argyrophilic proteins of the nucleolar organiser region at the optical level. Histochem J 18:5–14.

44. Mourad WA, Connelly JH, Sembra DL, Atkinson EN, Bruner JM (1993) The correlation of two argyrophilic nucleolar organizer region counting methods with bromodeoxyuridine-labeling index: a study of metastatic tumors of the brain. Hum Pathol 24:206–210.

45. Howell WM, Hsu TC (1979) Chromosome core structure revealed by silver staining. Chromosome (Berl) 53:37–50.

46. Plosten D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ (1986) Improvement in the staining and the visualization of the argyrophilic proteins of the nucleolar organiser region at the optical level. Histochem J 18:5–14.

47. Howell WM, Hsu TC (1979) Chromosome core structure revealed by silver staining. Chromosoma 21:61–66.

48. Li Q, Hacker GW, Danscher G, Sonnleitner-Wittauer U, Grimelius L (1995) Argyrophilic nucleolar organizer regions. A revised version of the Ag-NOR-staining technique. Histochem Cell Biol 104:145–150.

49. Rüschoff J, Prasser C, Cortez T, Höhne HM, Hohenberger W, Höfstadter F (1993) Diagnostic value of AgNOR staining in follicular cell neoplasms of the thyroid: comparison of evaluation methods and nuclear features. Am J Surg Pathol 17:1281–1288.

50. Lumachi F, Ermani M, Marino F, Polett A, Basso SM, Iacobone M, Favia G (2004) Relation-
Methods in neuroendocrine histopathology

ship of AgNOR counts and nuclear DNA content to survival in patients with parathyroid carcinoma. Endocr Relat Cancer 11:563–569.

49. Giuffrè G, Mormandi F, Barresi V, Bordi C, Tuccari G, Barresi G (2006) Quantity of AgNORs in gastric endocrine carcinoid tumours as a potential prognostic tool. Eur J Histochem 50:45–50.

50. Gomori G (1950) Aldehyde-fuchsin: a new stain for elastic tissue. Am J Clin Pathol 20:665–666.

51. Scott HR (1952) Rapid staining of beta cell granules. Stain Technol 72: 267–268.

52. Schiebler TH, Schiessler S (1959) Über den Nachweis von Insulin mit den metachromatisch reagierenden Pseudoisocyaninen. Histochemie 1:445–465.

53. Slidders W (1961) The OFG and BRAB-OFG methods for staining the adenohypophysis. J Pathol Bacteriol 82:532–534.

54. MacConaill MA (1947) Staining of the central nervous system with lead-haematoxylin. J Anat 81:371–372.

55. Falck B, Torp A (1961) A fluorescence method for histochemical demonstration of noradrenaline in the adrenal medulla. Med Exp Int J Exp Med 5: 428–432.

56. Falck B, Hillarp NÅ, Thieme G, Torp A (1962) Fluorescence of catecholamines and related compounds condensed with formaldehyde. J Histochem Cytochem 10:348–354.

57. Björklund A, Falck B, Owman Ch (1972) Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines. In: Rall JE, Koplin IJ (eds); The thyroid and biogenic amines. In: Book series: Berson SA (ed); Methods of Investigative and Diagnostic Endocrinology, vol 1. North-Holland Publishing Company, Amsterdam; pp 318–368.

58. Owman Ch, Håkanson R, Sundler F (1973) Occurrence and function of amines in endocrine cells producing polypeptide hormones. Fed Proc 32:1785–1791.

59. Björklund A, Falck B, Håkanson R (1968) Histochemical demonstration of tryptamine. Properties of formaldehyde-induced fluorophores of tryptamine and related indole components in models. Acta Physiol Scand, Suppl 318: 1–31.

60. Håkanson R, Sundler F (1971) Formaldehyde condensation. A method for fluorescence microscopic demonstration of peptides with NH2-terminal tryptophan residues. J Histochem Cytochem 19:477–482.

61. Björklund A, Falck B, Håkanson R (1968) Histochemical demonstration of tryptamine. Properties of the formaldehyde-induced fluorophores of tryptamine and related indole components in models. Acta Physiol Scand, Suppl 318:1–31.

62. Björklund A, Falck B (1969) Histochemical characterization of a tryptamine-like substance stored in cells of the mammalian adenohypophysis. Acta Physiol Scand 77: 475–489.

63. Larsson L-I, Sundler F, Grümelius L, Håkanson R, Bufià R, Solcia E (1975) Formaldehyde-ozone induced fluorescence in gastrin-producing tumours. Virchows Arch Path Anat Histol 365: 179–184.

64. Håkanson R, Sundler F, Larsson L-I, Ekman R, Sjöberg NO (1975) Peptides with NH2-terminal tryptophan in adrenocorticotrophic hormone and melanocyte-stimulating hormone granules of adenohypophysis. J Histochem Cytochem 23:65–74.

65. Larsson L-I, Sundler F, Håkanson R (1975) Formaldehyde-hydrochloric acid treatment. A fluorescence histochemical method for demonstration of tryptophan residues in peptides and proteins. J Histochem Cytochem 23: 873–881.

66. Larsson L-I, Sundler F, Håkanson R (1975) Formaldehyde-hydrochloric acid treatment. A fluorescence histochemical method for demonstration of tryptophan residues in peptides and proteins. J Histochem Cytochem 44:245–251.

67. Ehinger B, Thunberg R (1967) Induction of fluorescence in histamine-containing cells. Exp Cell Res 47:116–122.

68. Ehinger B, Håkanson R, Owman Ch, Sporrong B (1968) Histochemical demonstration of histamine in paraffin sections by a fluorescence method. Biochem Pharmacol 17:1997–1998.

69. Brody MJ, Håkanson R, Lundquist I, Owman Ch, Sundler F (1973) Cellular localization of glucagon by fluorescence microscopy. Reaction of NH2-terminal histidine with o-phthaldialdehyde. J Histochem Cytochem 21:13–16.

70. Brody MJ, Håkanson R, Owman Ch, Sundler F (1972) An improved method for the histochemical demonstration of histamine and other compounds producing fluorophores with o-phthaldialdehyde. J Histochem Cytochem 20:945–948.
71. Larsson L-I, Sundler F, Häkanson R (1975) Fluorescence histochemistry of polypeptide hormone-secreting cells in the gastrointestinal mucosa. In: Thompson JC (ed); Gastrointestinal Hormones. University of Texas Press, Austin; pp 318–368.
72. Häkanson R, Owman Ch, Sjölund K (1974) Cytospectrophotometric characterization of OPT-induced fluorescence in rat pinealocytes. Histochemistry 42: 15–23.
73. De Benardo S, Weigele M, Toome V, Manhart K, Leimgruber W, Böhlen P, Stein S, Udenfried S (1974) Studies on the reaction of fluorescamine with primary amines. Arch Biochem Biophys 163:390–399.
74. Häkanson R, Larsson L-I, Sundler F (1975) Fluorescamine: A novel reagent for histochemical detection of amine groups. Histochemie 59:15–23.
75. Larsson L-I, Sundler F, Häkanson R (1975) Fluorescamine as a histochemical reagent: demonstration of polypeptide hormone-secreting cells. Histochemistry 44:245–251.
76. Sundler F, Larsson L-I, Häkanson R, Ljungberg O (1974) Fluorescamine-induced fluorescence in C cell tumours of the thyroid. Virchows Arch A Path Anat Histol 363:17–20.
77. Coons A, Creech H, Jones RN (1941) Immunological properties of an antibody containing a fluorescent group. Proc Soc Exp Biol Med 47:200–202.
78. Coons AH, Leduc EH, Connolly JM (1955) Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J Exp Med 102:49–60.
79. Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG (1970) The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315–333.
80. Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques; a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29: 577–580.
81. Gould EA, Buckley A, Cammack N (1985) Use of the biotin-streptavidin interaction to improve flavivirus detection by immunofluorescence and ELISA tests. J Virol Methods 11:41–48.
82. Bobrow M, Harris T, Shaughnessy K, Litt GI (1989) Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. J Immunol Methods 125:279–285.
83. Heras A, Roach CM, Key ME (1995) Enhanced polymer detection system for immunohistochemistry. Mod Pathol 8: 163A.
84. Portela-Gomes GM (2005) Immunostaining techniques for co-localization of multiple peptide antigens in light microscopy. In: Hacker GW, Tubbs RM (eds); Molecular Morphology in Human Tissues: Techniques and Applications. In: Book series: Gu J, Hacker GW (series eds); Advances in Pathology, Microscopy & Molecular Morphology. CRC Press, Boca Raton; pp 1–26.
Methods in neuroendocrine histopathology

92. Shi SR, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39:741–748.

93. Feulgen R, Rossenbeck H. (1924) Mikroskopisch-chemischer Nachweis einer Nucleinsäure vom Typus Thymusnucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Preparaten. Hoppe-Seyler’s Z Physiol Chem 135:203–248.

94. Caspersson T, Lomakka G (1970) Recent progress in quantitative cytochemistry: Instrumentation and results. In: Wied GL, Bahr GF (eds); Introduction to Quantitative Cytochemistry, vol. II. Academic Press, New York; pp 27–56.

95. Burger G, Ploem JS, Goettler K (1987) Clinical Cytometry and Histometry. Academic Press, London; pp 1–550.

96. Shapiro HM (1989) Flow cytometry of DNA content and other indicators of proliferative activity. Arch Pathol Lab Med 113:591–597.

97. Bäckdahl M, Tallroth E, Auer G, Forsslund G, Granberg P-O, Lundell G, Löwhagen T (1985) Prognostic value of nuclear DNA content in medullary thyroid carcinoma. World J Surg 9: 980–987.

98. Cohn G, Erhardt K, Cedermark B, Hamberger B, Auer G (1986) DNA distribution pattern in intestinal carcinoid tumors. World J Surg 10:548–554.

99. Falkmer S, Erhart K, Auer G, Mårtensson H, Nobin A (1986) Patterns of DNA distribution and neurohormone immunoreactivity in the tumour cells; tools for the histopathological assessment of gastrointestinal carcinoids. Digestion 35, Suppl 1:144–152.

100. Bergholm U, Adami HO, Auer G, Bergström R, Bäckdahl M, Grimalius L, Hansson G, Ljungberg O, Wilander E (1989) Histopathologic characteristics and nuclear DNA content as prognostic factors in medullary thyroid carcinoma. Cancer 64:135–142.

101. Capella C, Solcia E, Frigerio B, Buffa R, Usellini L, Fontana P (1977) The endocrine cells of the pancreas and related tumours. Ultrastructural study and classification. Virchows Arch A Pathol Anat Histol 373:327–352.

102. Gall JG, Pardue ML (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci USA 63:378–383.

103. Burns J, Chan VT, Jonasson JA, Fleming KA, Taylor S, McGee JO (1985) Sensitive system for visualising biotinylated DNA probes hybridised in situ: rapid sex determination of intact cells. J Clin Pathol 38:1085–1092.

104. Bauman JG (1985) Fluorescence microscopical hybridocytochemistry. Acta Histochem, Suppl 31:9–18.

105. Brigati DJ, Myerson D, Leary JJ, Spalholz B, Travis SZ, Fong CK, Hsiung GD, Ward DC (1983) Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labelled hybridization probes. Virology 126: 32–50.

106. Varndell IM, Polak JM, Sikri KL, Minth CD, Bloom SR, Dixon JE (1984) Visualisation of messenger RNA directing peptide synthesis by in situ hybridisation using a novel single-stranded cDNA probe. Potential for the investigation of gene expression and endocrine cell activity. Histochemistry 81:597–601.

107. Raap AK, van de Corput MPC, Vervenne RAW, van Gijlswijk RP, Tanke HJ, Wiegent J (1995) Ultra-sensitive FISH using peroxidase-mediated deposition of biotin- or fluorochrome tyramides. Hum Mol Genet 4:529

108. Hacker GW, Graf A-H, Hauser-Kronberger C, Wirnsberger G, Schiechl A, Bernatsky G, Wittauer U, Su H, Adam H, Thurner J, Danscher G, Grimmelius L (1993) Application of silver acetate autometallography and gold-silver staining methods for in situ DNA hybridization. Chinese Med J 106:83–92.

109. Hacker GW, Hauser-Kronberger C, Graf A-H, Danscher G, Gu J, Grimmelius L (1994) Immunogold-silver staining (IGSS) for detection of antigenic sites and DNA sequences. In: Hacker GW, Gu J (eds); Modern Methods in Analytical Morphology. Plenum Press, New York; pp 19–35.

110. Hacker GW, Zehbe I, Hauser-Kronberger C, Gu J, Graf A-H, Grimmelius L, Dietze O (1995) Sensitive detection of DNA and mRNA sequences by in situ hybridization and immunogold-silver (IGSS). In: Gu J (ed); In Situ Polymerase Chain Reaction and Related Techniques for Morphologists. Eaton Press, Boston; pp 113–130.
111. Åkerström G, Pertoft H, Grimelius L, Johansson H (1979) Density determinations of human parathyroid glands by density gradients. Acta Path Microbiol Immunol Scand [A] 87: 91–96.
112. Åkerström G, Grimelius L, Johansson H, Pertoft H, Lundqvist H (1980) Estimation of the parathyroid parenchymal cell mass by density gradients. Am J Pathol 99:685–694.

Corresponding author:
Lars Grimelius, MD, PhD
Department of Genetics and Pathology
Unit of Pathology
University Hospital
75185 Uppsala
Sweden
Tel: +46 18 6113837
Lars.Grimelius@genpat.uu.se