西安交通大学

研究生创新教育系列教材

现代组织化学技术和方法

The Modern Technology and Method of Histochemistry

主编 周劲松

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The Modern Technology and Method of Histochemistry

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总序

创新是一个民族的灵魂，也是高层次人才水平的集中体现。因此，创新能力的培养应贯穿于研究生培养的各个环节，包括课程学习、文献阅读、课题研究等。文献阅读与课题研究无疑是培养研究生创新能力的重要手段，同样，课程学习也是培养研究生创新能力的重要环节。通过课程学习，使研究生在教师指导下，获取知识并理解知识创新过程与创新方法，对培养研究生创新能力具有极其重要的意义。

西安交通大学研究生院围绕研究生创新意识与创新能力改革研究生课程体系的同时，开设了一批研究型课程，支持编写了一批研究型课程的教材，目的是为了推动在课程教学环节加强研究生创新意识与创新能力的培养，进一步提高研究生培养质量。

研究型课程是指以激发研究生批判性思维、创新意识为主要目标，由具有高学术水平的教授作为任课教师参与指导，以本学科领域最新研究和前沿知识为内容，以探索式的教学方式为主导，适合于师生互动，使学生有更大的思维空间的课程。研究型教材应使学生在学习过程中可以掌握最新的科学知识，了解最新的前沿动态，激发研究生科学研究的兴趣，掌握基本的科学方法，把教师为中心的教学模式转变为以学生为中心教师为主导的教学模式，把学生被动接受知识转变为在探索研究与自主学习中掌握知识和培养能力。

出版研究型课程系列教材，是一项探索性的工作，也是一项艰苦的工作。虽然已出版的教材凝聚了作者的大量心血，但毕竟是一项在实践中不断完善的工作。我们深信，通过研究型系列教材的出版与完善，必定能够促进研究生创新能力的培养。

西安交通大学研究生院

Preface

The composition of *The Modern Technology and Method of Histochemistry* which supported by Xi'an Jiaotong University, is under supervision of highly experienced professors. The main purpose of this textbook is to cultivate basic experiment skills, enhance research activity and innovative spirit.

Histochemistry, which widely used in life science, is basic science and technology for medical graduate students. In order to make sure the graduate students understand and master basical knowledge and methods, different experiments and corresponding images are added to demonstrate the theories. Since Histochemistry is also fast-developing science, advanced methods in fixation, new fluorescence dyes as cell markers, the use of flow cytometry confocal laser scanning microscope and other new technologies are emphasized. The photomicrography is also introduced as well.

This textbook is suitable for foreign medical graduates in preclinical Medicine and Clinical Medicine, and can also be used as references for other fields, such as Preventive Medicine, Nursing and Pharmacy.

Acknowledgments must be made to Professor Shudong Qiu, Professor Tianbao Song, Professor Zhen Li, Ming Li, Lirong Wang, editor Yincun Wang, Huali Wang and Xuanjing Du for their kind help. There might be some omissions and mistakes due to time limition, all the corrections and suggestions are highly welcomed.

Jinsong Zhou
Xi'an Jiaotong University
March, 2014

Contents

Chapter 1 Introduction

The development of life sciences requires deep understanding of chemical compositions, their structural and functional relationships. During the past hundreds of years, more and more theories, technologies and methods were created and developed to detect the chemical compositions in tissue and cells, and a borderline discipline, the Histochemistry, were established. As a methodology, nowadays Histochemistry is widely used in basic life science research and clinical application.

Section 1 The contents and theories in Histochemistry

I. The arise and development of Histochemistry

Histochemistry is also known as micro-chemistry, which means the reaction processes and results can not be observed with naked eyes or in test tube but under microscope. At the very beginning, the chemistry methods just occupied a little in Histochemistry contents due to insufficient of chemistry knowledge. In the 20th century, the eager to know the nucleus acids, the proteins and the enzymes requires the developrent of Histochemistry, more and more new methods were created and verified. For example, the Feulgen reaction and the methylene green-pyronine were used to display the nucleus acid, PAS reaction was introduced to show glycogens, and the calcium-cobalt and lead nitrate methods were applied to demonstrate the alkaline phosphatase and acid phosphatase, respectively. The Immunohistochemistry method was created when specific antigen-antibody reaction was used to detect the antigen compositions in tissues and cells. The molecular biology technologies, such as in situ hybridization and PCR, were introduced to combine with Histochemistry. Meanwhile, the quantitation assay technologies, such as photomicrography, image analysis, flow cytometry and laser scanning confocal microscopy were established and took in application.

Thus, as a newly established borderline discipline, Histochemistry knowledge contains the information in Histology and related lasted methods, advanced technologies and discoveries. So the concept of Histochemistry should be like this: based on Histology, the modern methods and technologies in Physics, Chemistry, Biochemistry, Immunology and Molecular Biology are introduced to detect the chemical compositions in situ, and the qualitative and quantitative analysis are carried on to understand the normal and abnormal rules about the metabolisms, functions and morphology changes in cells and tissues.

II. The contents of Histochemistry studies

1. The inorganic materials in cells

Inorganic materials in cells and tissues mainly include different metal ions, such as Ca^{2+}, Mg^{2+}, K^{+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Pb^{2+}, Ag^{+}, Au^{3+}, As^{3+}, U^{6+} and I^{-}, et al, as well as their salts, such as chloridate, phosphate, carbonate and nitrate, et al.

2. The organic materials in cells

The organic materials in cells and tissues mainly include sugar (glycogen, starch, glycoprotein and proteoglycan, etc), lipid (such as phospholipid, glycolipid, lipoprotein, lipoid, cholesterol and sterol ester, etc), nuclear acid (DNA and RNA), peptide, protein, pigments and different kinds of vitamins.

3. The different kinds of enzymes

As special proteins, the enzymes play important roles in cell metabolism. Today, more than 200 kinds of enzymes can be displayed through Histochemistry methods, such as acid phosphatase, alkaline phosphatase, 5-nucleotidase, glucose-6-phosphatase, adenosine triphosphatase, adenosine triphosphatase, carbonic anhydrase, non-specific esterase, cholinesterase, cytochrome oxidase, peroxidase, monoamine oxidase, succinate dehydrogenase, lactate dehydrogenase, 3β-hydroxy steroid dehydrogenase, acylase and phosphorylase, etc.

4. The antigen and antibody in cells and tissues

The specific antigen-antibody reaction can be used to detect not only the antigens in tissues or antigens from pathogenic microorganism, but also autoantibody and antibody-antigen complex, and provide reliable results for basic research and clinic diagnosis.

5. The endogenous and exogenous gene segments

With or without PCR technology, the in situ hybridization technology can be used to detect endogenous gene segments and their normal and abnormal expression, such as DNA and mRNA, and gene segments of virus which can be used as references for clinical diagnosis.

III. The theories of Histochemistry

1. Chemistry reaction

The already-known chemical reactions are used to demonstrate the materials in tissues and cells by forming colorful deposits or high electron density structures through one or several steps, such as enzyme Histochemistry. Firstly, the chemicals used for detection will be structurally changed in situ by the target materials; secondly, additional chemical regents are applied to indirectly demonstrate the target materials in cells and tissues, such as PAS and Feulgen reaction.

2. Physics theory

Certain materials can be visualized by their physics characteristics, for example, fat can be dyed by colorful Sudan series dyes because the dyes are lipid-soluble, and the florescence can be observed when monoamine (such as noradrenaline, dopamine and 5-hydroxytryptamine, etc) is induced by formaldehyde under the fluorescence microscope.

3. Biological characteristic

Most biomacromolecules can be regarded as antigens, and these antigens will combine and show the clour that their specific antibodies be marked with, such as fluorescence, enzymes or colloid gold. Some affinity chemical reactions, such as avidin and biotin has becvme a branch of Histochemistry, the affinity Histochemistry.

4. Nucleotide chain complementary principle

The two complementary nucleotide chains will combine with each other to form stable hybrid. When one of them is labeled with a marker, it can be used in the in situ hybridization to show the (target) chain.

Section 2 The basic requirements of Histochemistry methods

Different theories, procedures and results match different Histochemistry methods, but they all have the same purpose: to display the tissue and cell chemicals in situ. Thus, basic common requirements must be followed.

I. Basic requirements

1. The specificity

High specificity of reactions against the target materials guarantees the right experiment results.

2. The sensitivity

High sensitivity of reactions assures that the method can be used to detect trace target materials in cell and tissue.

3. The fixation

Perfect fixation will provide authentic cell, tissue and material structures for histochemistry staining, which is necessary for in situ observation and records.

4. The reaction deposits

The reaction deposits must be formed in situ, and should be insoluble, stable and colorful for light microscopy or with high electron density for electronic microscopy.

5. The repeatability

The repeatability is basic rule for all kinds of scientific researches.

II. Things need to know

1. The characteristics of target materials, such as water-solubility, lipid-solubility and the possible location in cell and tissue should be clear before experiment. This means many references must be thoroughly read and understood.

2. The procedures of experiments must be strictly controlled, such as the concentration of every chemical reagent, the temperature and pH value of reaction liquid, especially for enzymes. In the control test and following repeated experiments, conditions should remain the same.

3. There should be control tests for each experiments. Positive control test is

used to verify the experiment method, procedures and the reagents, and negative test is used to demonstrate the specificity of experiment results, which is crucial for result analysis.

4. All reagents applied in experiments should be analytical pure (A. R class), and hould not influence the chemical characteristics of target materials or the activity of enzyme.

5. All the lab utensils should be clean. The water in expariments should be double-distilled water (DD H_2O).

Questions

Generally, what are the differences of study fields among Histology, Histochemistry and Immunohistochemistry?

(Jinsong Zhou)

Chapter 2 The Tissue Preparation

In general, the specimen could be studied with histochemistry and immunohistochemistry method only after the processes of tissue collection, fixation, embedding and sectioning have been taken.

Section 1 Tissue collection

I. Points for attention

1. Keep the instruments sharp and clean.
2. Collect the sample quickly and accurately.
3. Keep the sample from artificial damage.
4. It's better to collect the tissue at low temperature (0℃-4℃).

II. The size of specimen

The specimen should be small and thin so that the fixative permeates the sample slowly. In general, the size of specimen for light microscope is about 1cm ×1cm×0.5cm, and $1mm^3$ for most electron microscope study.

Section 2 Fixation

The purpuse of fixation is preserve the activity of tissue structure and chemical compositions as far as possible. Specimens should be fixed immediately after they are cut from the body.

I. The purpose of fixation

Fixation preserves the tissue and cell in living condition as much as possible for subsequent treatment. The effect of fixation including:

1. Inhibit autolysis

After the tissue is isolated from body, the lysosome breaks and releases lyso-

somal enzymes because of hypshia, which results cell damage. This process is known as autolysis. Fixative can inactivate lysosomal enzymes.

2. Prevent solution

Tissue compositions are dissolved during tissue preparation because most reagents can dissolve chemicals in tissues.

3. Avoid corruption

Fixative can fix proteins to kill bacteria.

4. Reduce injury

Fixation can make the cytoskeletal proteins more stable.

5. Harden the tissue

Fixative can harden the soft tissue, which is beneficial for sectioning.

6. Alter refractive index

Fixation can change the refractive index of cell and tissue composition to some extent, which makes the structures to be discerned easier.

7. Enhance the dyeing

In a sense, fixative possesses mordant dyeing effect to enhance the dyeing.

II. Object for fixation

The fixatives may react with proteins in following ways:

1. The chemical bonds between fixative and protein form precipitates.

2. Fixatives interrupt the degeneration of protein. Protein will lose solubility after degeneration.

Basically, other materials such as lipid, sugar and sugar-like chemicals, nucleic acid and other large molecules are combined with different kinds of proteins, and they can be fixed by protein-fixation processes.

III. The quality of fixative

The fixatives usually should possess the following qualities:

1. Fixatives penetrate the tissue and cell quickly, but do not change the tissue structure.

2. Fixatives produce tissue swelling and contraction as less as possible.

3. Specimen could be saved in the fixatives for a relatively long period.

IV. Method for tissue fixation

1. Soak fixation

Soak fixation is also called immersion fixation. In this kind of fixation, the following steps are followed: Keep the fixation in 0℃ - 4℃; Place several layers of gauze and absorbent cotton at the bottom of glass container; and then, put the specimen in fixative for several hours at least.

2. Perfusion fixation

Fixative is perfused through the whole body or an organ by cardiovascular system, in this way, the living cells are fixed in situ quickly. Buffer or normal saline containing proper amount of heparin is used to treat the blood before fixatives are injected into the cardiovascular system. The amount of fixatives and the perfusion pressure are different according to different animals. Tissue is collected after perfusion fixation and immersed into the same fixative if necessary.

3. Cultured cells fixation and smear fixation

As for monolayer culture, remove the coverslip from culture plate, and perform fixation. As for suspension culture, prepare smear after concentration by centrifuging. Then, the smear is fixed in fixatives. Usually the glass slice needs to be coated by adherence chemicals before using to prevent tissue slice shedding.

V. Notes for fixation

1. Fixed specimen must be fresh. The specimen should be fixed immediately after removed from the animals. Long time delay will result tissure shrinkage or autolysis, which is big taboo of Histochemistry and Immunohistochemistry.

2. Fixed specimen should be put into sufficient quantity of fixative. The general fixation dose is 20 to 50 times size of tissue block, too insuffficient fixatives should be avoided, and on the other hand, too much fixative is not necessary.

3. Fixation duration time depends on the tissue type, size, and the kind of fixatives. Generally, 24 hours is enough for tissue block, and 15 seconds for cultured cells in one layer on glass slide.

4. In general, specimen can be fixed at room temperature. Fixative for enzyme detection should be placed in refrigerator before using.

5. The selection of fixative depends on the type of tissue. Before the Histo-

chemistry and Immunohistochemistry experiments, the selection of fixatives is tricky, especially for new tissue or new materials which will be detected. In this case, reading more references and doing more pre-experiments are strongly recommended.

VI. The frequently used fixative

Fixative can be divided into the following types according to their chemical characteristics:

Type Ⅰ: Aldehyde, such as formaldehyde, glutaraldehyde, paraformaldehyde, acrylaldehyde, malondialdehydes, and so on.

Type Ⅱ: Oxidant, such as osmic acid, kalium hypermanganicum ($KMnO_4$), bichromicum kalium, and so on.

Type Ⅲ: Protein denaturation agent, such as methyl alcohol, alcohol, acetic acid, and so on.

Type Ⅳ: Others, such as mercuric chloride, picric acid, and so on.

According to the usage methods, fixative can be used solo or with other fixative(s) as a mixture. So, they can also be divided into pure and mixture fixatives.

1. The pure fixative

Commonly used pure fixative includes formaldehyde, alcohol, glacial acetic acid, picric acid, bichromicum kalium, osmic acid, mercuric chloride, acetone, and so on. Formalin, alcohol and acetone are commonly used as pure fixatives; others are used as an ingredient of mixed fixatives.

1.1 Formaldehyde Formaldehyde is a gas, and its saturated water solution which contains 37%-40% formaldehyde is named formalin. Generally, 10% formalin (4% formaldehyde solution) is used as fixative. Formaldehyde is also stable as solid form named paraformaldehyde, the high molecular weight polymer.

Formaldehyde is a reducing agent. In water, formaldehyde monomer is a monohydrate namely methylene glycol ($CH_2(OH)_2$). It is the most effective fixative. Formaldehyde connects the adjacent proteins with bridging bonds, and become insoluble polymer. Common formaldehyde reaction is that it is added to a reactive hydrogen compounds and forms methylol compounds. Any further condensation forms methylene bridges with other hydrogen atoms. The equation is as follows:

$$RH + CH_2O \rightleftharpoons R \cdot CH_2(OH)$$