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Chapter 1

Introduction

One of the most dramatic changes in my 30 + year career has been the explosion of the field of molecular pathology. Technological changes have resulted in the evolution of a field that basically did not exist 20 years ago, to the point that it is now a dominant player in both research and clinical medicine. There are two in situ-based tests: in situ hybridization and immunohistochemistry. Indeed, in clinical pathology, many diagnoses are dependent on performing an in situ-based test. Further, clinicians often depend on specific in situ-based results to make the definitive decision on how to treat a patient's disease. For example, the her-2-neu immunohistochemical test is routinely done to decide whether a woman's breast cancer will show reduced growth if treated with a specific drug called Herceptin that has minimal side effects, at least relative to standard chemotherapy.

The critical need for immunohistochemistry and in situ hybridization tests in the diagnostic and research arenas has led to the involvement of major biotechnical companies in this area. Large companies such as Enzo Biochem, Roche, Ventana Medical Systems, Dako, Leica, and others offer many products for in situ-based molecular pathology including automated platforms, and they, and many other large companies, market reagents for such tests. This has led to basically all diagnostic and most research laboratories using these automated in situ hybridization- and immunohistochemical-based systems. I can attest that 25 years ago the idea that automated machines could do in situ hybridization and immunohistochemistry was almost in the realm of science fiction!

As a result of these advances in the field of in situ-based molecular pathology, many more laboratories are either using these tests for their diagnostics or research, or wanting to incorporate them into their work. Hence, the purpose of this textbook. I hope that this book can make in situ-based molecular pathology more accessible and understandable to both the research and diagnostic laboratory. I hope to do this by focusing on two key goals: (1) to explain the theory and foundation of immunohistochemistry and in situ hybridization and (2) to present simplified protocols that are easy to follow for the different in situ-based protocols. I also include protocols for the identification of two or more DNA/RNA/protein targets in a given tissue.

This textbook has been written assuming a minimal prior knowledge of the topics of molecular pathology in general and in situ-based molecular pathology in particular. The first chapters focus more on the biochemistry of the processes inherent in any molecular pathology-based method, including the polymerase chain reaction (PCR) and hybrid capture solution phase detection of DNA or RNA, as well as Western blot detection of proteins. The biochemistry part, though, strongly emphasizes just the key parts you must understand to be able to "visualize" what is actually happening inside the intact cell when doing either immunohistochemistry or in situ hybridization. As all such methods, of course, use intact tissue, I also include a chapter to assist you in being better able to determine the cell type(s) that contain the target sequence of interest. Specifically, I include a chapter that is meant to teach the basics of histopathology to the nonpathologist. This second edition includes a thorough quiz on the interpretation of basic histopathology in the Appendix, which I hope will help the reader with little experience in this area become more adroit at examining tissue under the microscope. The second edition also includes two new chapters. One deals with differentiating signal from background. In this chapter, one will see that by combining their knowledge of histopathology with the color-based changes of the in situ molecular tests, they will rarely (hopefully, if ever) misinterpret background as signal. The other new chapter focuses on several major developments in the fields of in situ hybridization and immunohistochemistry that have been described since the first edition was published. After this basic introduction to these key topics, we move on to the practical applications of in situ hybridization, immunohistochemistry, and coexpression analyses.

Thus, it is certain that all readers will be able to either just breeze through or skip certain sections, depending on your training. It is my strong hope that all readers, after finishing this book, will not only want to try their hand at

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in situ-based molecular pathology but also have the confidence that they will be able to reason out the best way to solve the problems that arise when using any such methodologies. The end result, I hope, will be well worth the effort. For one, the power of the in situ-based molecular pathology tests is extraordinary. By knowing the cell type or types that contain the target of interest, you typically get tremendous insight into the role of the target that simply cannot be achieved by PCR, Western blots, or any of the other solution-based methods, as each of the latter tests requires the pulverization of the tissue as a prerequisite to doing the test. Also, with these methods, you can get the true pleasure of looking under a microscope and often seeing for the first time data that no one before has seen, especially when working with novel DNA/RNA or protein sequences. Thus, we can appreciate the wonder and excitement of Van Leeuwenhoek when he first examined microbes under the microscope. The fun and enjoyment of doing this is why I enjoy in situ hybridization and immunohistochemistry today every bit as much as, if not more so, when I started 30 years ago!

When I started writing this book, I realized that I had certain preconceived notions about in situ hybridization and immunohistochemistry. It seemed that the format of writing a book in this field was the perfect time to test such preconceived notions. For example, I had assumed for my entire career that if I was unable to get a good signal for either immunohistochemistry or in situ hybridization with an older block (usually defined as at least 10 years old), the target DNA/RNA/protein had simply degraded and that was that. I was trained (and it made perfect sense) to simply avoid such blocks of tissue, as they probably were not fixed properly at the time of biopsy and, more importantly, nothing could be done to "rejuvenate" the signal. Similarly, I was trained to assume that any RNA would quickly degrade in the tissue sections, either from just time-related degradation and/or RNase activity in the tissue/in situ solutions and, thus, to only use recently done formalin-fixed biopsies for RNA in situ hybridization analysis, and to also use strict "RNase-free" protocols. Although I could give you many such other examples, let me end with just one more. I was trained to rely primarily on one method to "expose the target" when doing in situ hybridization or immunohistochemistry. This method has many names, including *antigen retrieval, cell conditioning*, and *liquid-based denaturation.* Again, this made perfect sense because it was well documented that formalin fixation cross-linked cellular proteins to each other and to RNA/DNA. The logic went that this extensive cross-linking created many small pores that needed to be opened for the DNA/RNA probe or primary antibody and ancillary reagents to enter the cell and access the target. Of course, this theory became very popular when antigen retrieval first came on the scene about 25 years ago, and many proteins that were otherwise undetectable with immunohistochemistry became evident. Certainly, I clearly remember the importance of antigen retrieval to the anatomic pathologist in breast cancer, as the ER/PR and her-2-neu testing required this pretreatment to get an accurate idea of the signals that, in turn, had important implications for the treatment of the woman.

An important focus of this book is that all the preconceived notions noted in the preceding paragraph, despite making sense, are simply wrong! Look at Fig. 1.1. This is the result of in situ hybridization for HPV DNA in a tissue sample over 20 years old. When I first tested the block in 1992 (just when HPV could be successfully detected in situ), it produced an intense signal, as seen in panel A. When I tested the same tissue in 2012, basically no signal was evident (panel B). Again, I just simply assumed that the HPV DNA had degraded over time and probably simply diffused out of the cell. I also assumed that this block was therefore worthless for any further

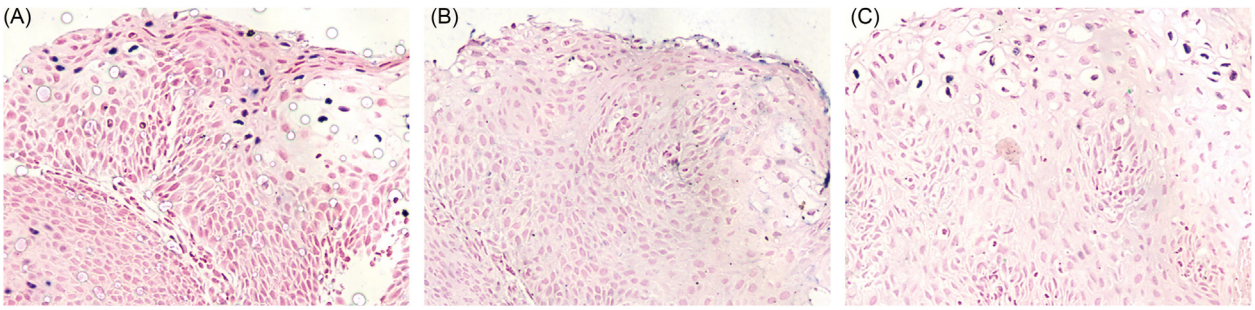

FIGURE 1.1 Effect of the tissue block age on the HPV in situ hybridization signal and its "rejuvenation." Panel A shows the intense signal for HPV in situ hybridization in this cervical intraepithelial lesion grade 1 (CIN 1) obtained in 1992. Serial section slides were saved for 20 years. In 2012, when the serial section was tested for the same HPV type (HPV 51), the signal was lost (panel B). However, when another serial section was treated with a series of reagents meant to regenerate the signal, the intense HPV 51 signal returned and was, thus, rescued (panel C). We discuss in detail the "regeneration of the signal" concept in subsequent chapters. But, for now, these data show that such aged blocks still can be useful for in situ hybridization-based research.

DNA or RNA testing, and probably for any protein testing by immunohistochemistry as well. But look at panel C. This is a serial section of the same tissue. I treated the tissue with a "rejuvenating" agent and then did the in situ hybridization. The signal was beautifully regenerated! Fig. 1.2 shows the exact same situation for a protein (cytokeratin AE1/3) in a block of tissue 20 years old. Fig. 1.3 shows the same result for RNA, in this case, microRNA-let-7c. Clearly, the idea that DNA, proteins, and, especially, RNA degrade over time in formalin-fixed tissue and that this per se precludes their detection by in situ hybridization or immunohistochemistry is simply wrong! And, again, this is what I was taught and I certainly believed for many years.

We spend several of the next chapters on understanding the reason that you can use old tissue blocks and slides with immunohistochemistry and in situ hybridization and, by understanding what is happening to the tissues and macromolecules over time, regenerate the signal and basically make the tissue not only as good as new but, in most cases, "better than new." This leads to a fundamental part of this book. Recipes (often called cookbook recipes) for

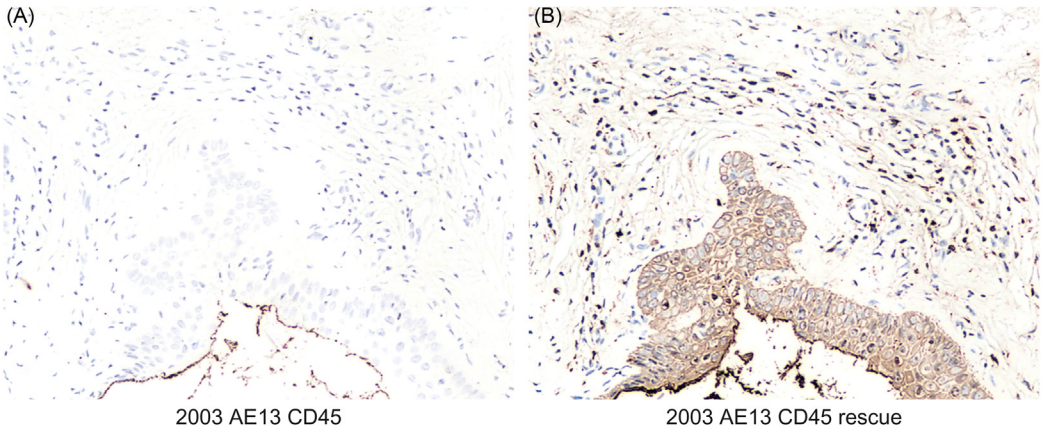

FIGURE 1.2 Effect of the tissue block age on the immunohistochemistry signal and its "rejuvenation." Panel A shows results of immunohistochemistry for two proteins, cytokeratin AE1/3 and CD45, in a skin biopsy of a patient with nonspecific dermatitis. The biopsy was done in 2003, and serial sections saved for the last 9 years. Note the very weak signal for the cytokeratin and the lack of a signal for CD45 in the lymphocytes that are present in the dermis. Both proteins should yield intense signals in such a biopsy. An additional serial section slide was treated with the same series of reagents meant to regenerate the signal as used for the HPV test in Fig. 1.1. Note that the intense signals for each cytokeratin and CD45 are now evident (panel B). We discuss in detail the "regeneration of the signal" concept in subsequent chapters. But, for now, these data show that such aged blocks still can be useful for immunohistochemistry research.

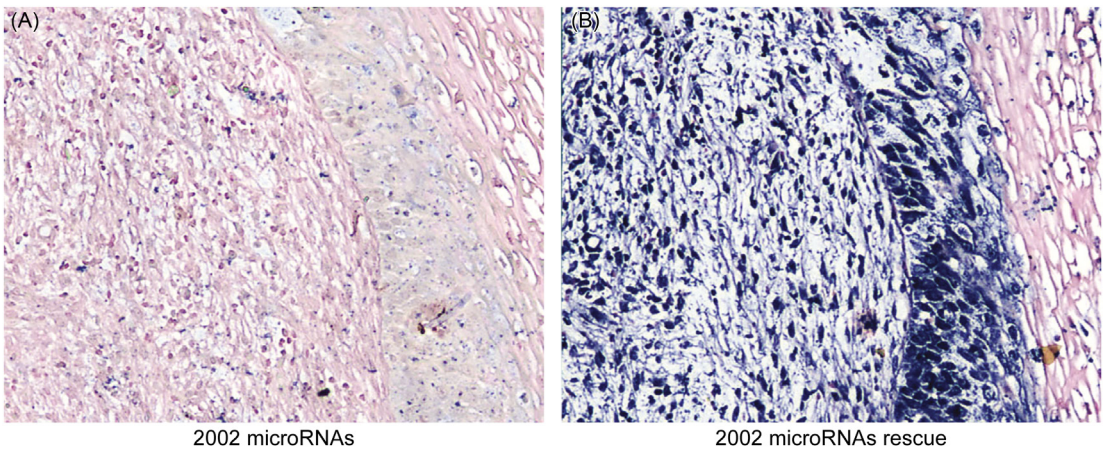

FIGURE 1.3 Effect of the tissue block age on the in situ hybridization signal for microRNAs and its "rejuvenation." Panel A shows a cervical biopsy with nonspecific inflammation taken in 2002. Unstained slides were stored for 10 years. The tissue was tested for miR-31 and miR-let-7c. These miRNAs should be present in high copy number in the cervix in the stromal inflammatory cells and basal epithelial cells, respectively. However, no signal was noted. An additional serial section slide was treated with the same series of reagents meant to regenerate the signal as used for the HPV test in Fig. 1.1 and the immunohistochemistry test in Fig. 1.2. Note that the intense signals present in the submucosal inflammatory cells and basal epithelial cells are now evident (panel B).

in situ hybridization and immunohistochemistry are helpful; indeed, they are essential because they serve as a starting point for these methods. However, I do *not* want this book to only give you such recipes. I think it is essential that we all, to the best of our ability, understand the biochemistry of each step of immunohistochemistry and in situ hybridization. This requires an in-depth knowledge of what actually happens at a biochemistry level inside the intact cells when we use cryostat sections versus denaturing fixatives (such as ethanol, acetic acid, and alcohol) versus the most common fixative, 10% neutral buffered formalin. This knowledge will be *by far* the most important tool you will have to troubleshoot when you are experiencing problems with immunohistochemistry and in situ hybridization.

Now, look at Fig. 1.4. These are all images of HPV in situ hybridization. Note that in some of the tissues the optimal signal requires DNA retrieval. By "DNA retrieval," I mean exposing the tissue to 95°C in an aqueous solution before in situ hybridization (like antigen retrieval for proteins with immunohistochemistry). However, you will see tissues that are histologically equivalent will not give a good signal with "DNA retrieval" but rather require protease digestion for an optimal HPV in situ hybridization. You will also see, as illustrated in panels C and D, cases in which tissues with the same histologic diagnosis require *no* pretreatment to get the best signal. In yet other tissues, antigen retrieval *plus* protease digestion gives the best signal! I can assure you that it is impossible to predict which HPV-infected formalin-fixed, paraffin-embedded tissue will

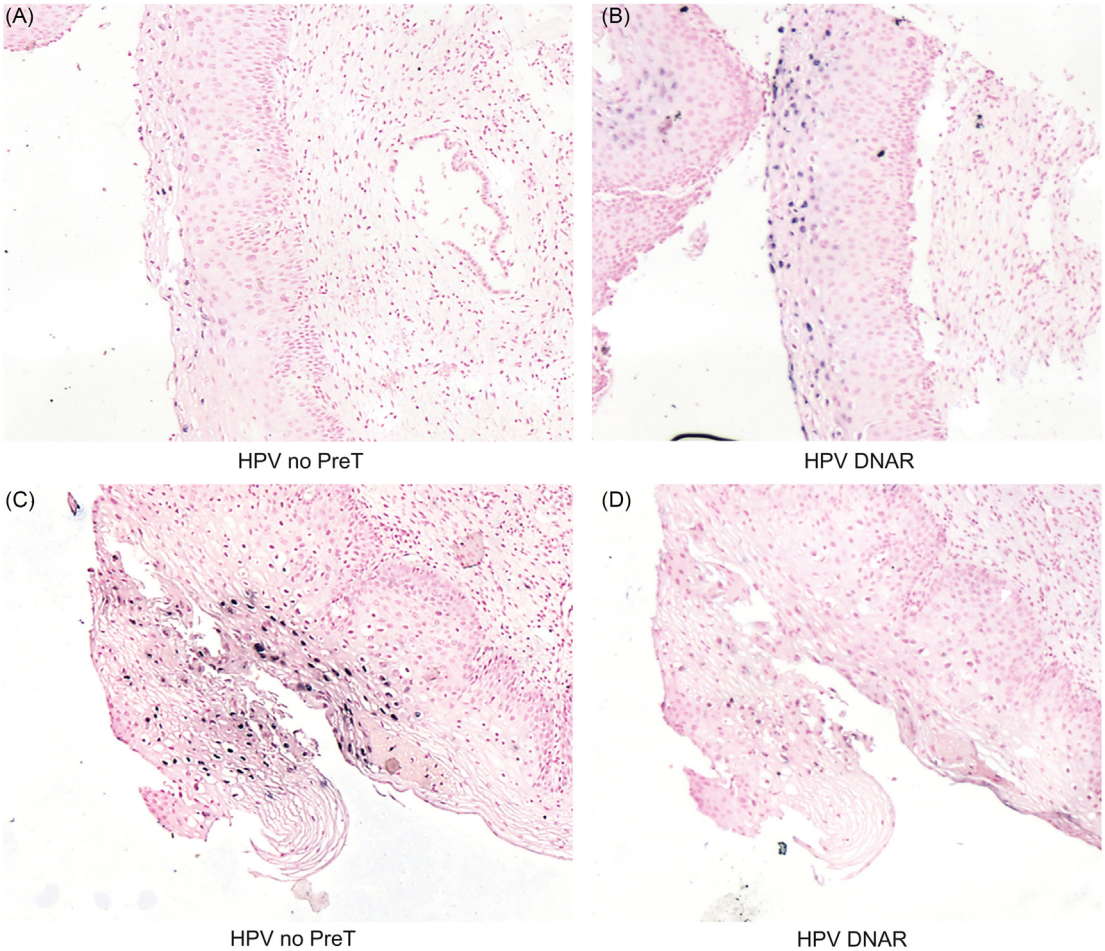

FIGURE 1.4 Different optimal protocols for HPV in situ hybridization for different CIN tissues. Each tissue was diagnosed as CIN, and each was obtained in 2011 or 2012. Note that one of the biopsies showed a very weak signal with no pretreatment (panel A), but when a serial section was incubated at 95°C for 30 min in an EDTA solution, the signal became much stronger (panel B). However, note that a different CIN tissue, which looked equivalent to the CIN shown in panels A and B, yielded the exact opposite results. Specifically, there was a strong signal with no pretreatment (panel C) that was much reduced when the serial section was incubated at 95°C for 30 min in an EDTA solution (panel D). These data underscore the important point that tissues from the same site with the same diagnosis may well require different pretreatment conditions when doing in situ hybridization for RNA or DNA.

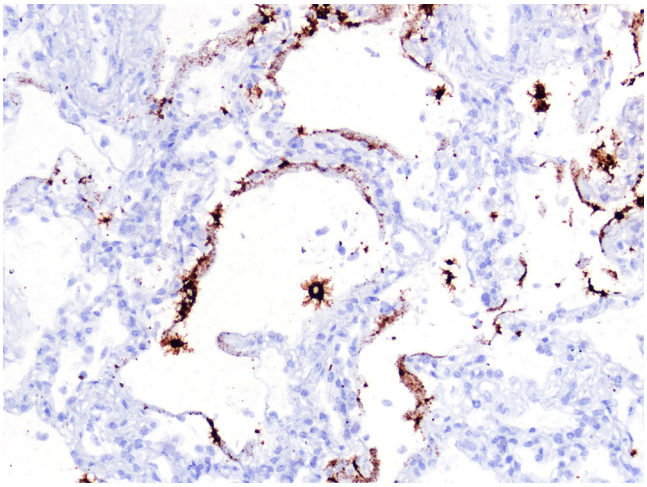

FIGURE 1.5 Detection of SARS-CoV-2 RNA by in situ hybridization. Note the strong signal for the viral RNA in this lung from a person who died of COVID-19. Viral RNA is seen in the stellate macrophages and the endothelia of the septal capillaries.

require no pretreatment, antigen retrieval, protease, or a combination of the last two pretreatment regimes. It is important to stress that if you do not use the right pretreatment regime for that given tissue, then you might not see *any* signal. Why does this happen? What is the biochemical basis of this observation? We discuss this topic at length in the book and, I hope, by the time you reach the end with Chapter 12, PCR In Situ Hybridization and RT In Situ PCR, you have a solid understanding of this and related phenomena.

I mentioned above the excitement of seeing for the first time under the microscope a specific RNA, DNA, or protein detected in situ. Since we are in the midst of the worst pandemic of the last 100 years with SARS-CoV-2 (COVID-19), I would be remiss if I did not include a photo of this virus. Figure 1.5 shows the very high amount of SARS-CoV-2 RNA present in the lung of someone who died of this horrible disease. In my opinion, the answer to how people die of COVID-19 lies to a large degree in the in situ based methods that detect the virus and correlate its presence to the host response.

The preceding paragraph, where it is clear that one pretreatment regime may be perfect for one tissue and give no signal at all for another tissue with the same pathologic diagnosis, may be a bit disheartening to the beginner. Not to worry! We discuss in detail the biochemical basis for this observation and learn how to use it to our advantage when we devise our in situ hybridization and immunohistochemical-based protocols. So, let's begin with a discussion of some of the basic concepts of molecular biology in Chapter 2, The Basics of Molecular Pathology.

Before we move to Chapter 2, The Basics of Molecular Pathology, let's take a quantitative look at how the fields of in situ hybridization and immunohistochemistry have grown over the past 3 decades. The suggested readings show the number of publications produced in 1975 on the topic of in situ hybridization or immunohistochemistry; note that there were eight such papers [1–8]. Compare this to the list of publications produced in 1980 on in situ hybridization or immunohistochemistry; note that there were 34 [9–42]. These references are included to give homage to the pioneers in these two fields.

Let's now look at the number of peer review references on either in situ hybridization or immunohistochemistry over the past 35 years. These data are presented in the following table.

Year	Number of peer review papers on either in situ hybridization or immunohistochemistry
1975	8
1980	34
1985	150
1990	5030
1995	11,023
2000	12,216
2005	16,610
2010	13,337
2015	12,561

As is evident, an explosion of papers on in situ-based molecular pathology was published between 1985 and 1995. In 1995, the field became firmly established in biomedical research and diagnostics.

Suggested readings

Publications on in situ hybridization and immunohistochemistry in 1975

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