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Coexpression analyses

Introduction

One of the most exciting developments with in situ hybridization and immunohistochemistry over the past 5 years is the rapid development of coexpression analyses. As a pathologist, I always find it exciting to see a well-done coexpression analysis slide, because it provides so much information about the interplay of the two targets of interest. The fuel for the development of the field in the past few years has certainly been the explosion in the interest in the biological properties of microRNA (miRNA) molecules.

If you are my age or older, you may remember a term we heard a lot in molecular biology classes 30–35 years ago. That term was “junk DNA.” Most of us may recall when the professor indicated that a high percentage of human DNA had no coding function and, thus, was “junk DNA” because it was not doing anything “important.” In this era of investments called “high quality” that in reality are junk, it is nice to see that nature reversed that situation and made “junk DNA” into one the most important and valuable discoveries of the past 10 years—noncoding RNA—although, admittedly, this is just one part of “junk DNA.”

As the name implies, noncoding RNAs do not generate proteins per se. However, they can have profound effects on the function of key mRNAs and proteins. One of the most important families in the overall class of noncoding RNAs is the miRNAs. As of the writing of this book, 1921 mature miRNAs have been identified in humans. It is estimated that the majority of all metabolic pathways in normal physiology, as well as oncogenesis, are directly impacted by microRNAs.

miRNAs function mainly by sharing sufficient homology with the 3-untranslated region (UTR) of a given mRNA. By binding to the UTR, the miRNA can either stop (or slow down) the transcription of the mRNA to a protein, or it can lead to the mRNA’s complete degradation. Either way, the miRNA can effectively eliminate the mRNA from the cell as effectively as a mutation. miRNAs are, as their name implies, very small molecules of typically 20 nucleotides in size. Since typically only 7–8 of their nucleotides participate in the binding of the UTR of the target mRNA, we can easily see from our discussions of the melting curve (Tm) that such hybridizations may be fraught with a strong propensity to denature. To “compensate” for this relatively weak force of hybridization, miRNAs have seemed to develop two other features that are important relative to their detection with in situ hybridization:

1. They tend to be in very high copy number when physiologically active.
2. They tend to be strongly associated with multiple different proteins, including the Argonaute proteins that are part of the RISC (RNA-induced silencing complex) complex.

Of course, point 1 works very much to our advantage when doing in situ hybridization. This is the same reason that it usually is very easy to detect DNA viruses by in situ hybridization or immunohistochemistry. The reason is that DNA viruses such as herpes simplex, human papillomavirus, Epstein–Barr virus (EBV), and cytomegalovirus produce large amounts of their DNA, RNA, and proteins. As miRNAs are so small and, thus, would have a low melting temperature in nature, it seems logical to assume that this “primitive” system of mRNA control evolved to be associated with very high copy number when physiologically active. Whatever the reason, this will be enormously advantageous to us when we do in situ hybridization for miRNA.

Point 2 is an interesting point. It has been well established that miRNAs are strongly bound to a variety of proteins that play key roles in “presenting” and “docking” the miRNA to the mRNA target. The multiple protein “cage” associated with microRNAs is called the RNA-induced silencing complex (or RISC for short). It uses the mature miRNA as the template for recognizing the mRNA to which the miRNA has homology in the 3-UTR region. When this happens, as indicated previously, either RNase is activated and the mRNA is destroyed, or the transcription process slows down dramatically, effectively eliminating the physiologic role of this mRNA. It is
interesting to speculate that the dense protein cage that surrounds miRNAs in the RISC may help compensate for the relatively weak homology between the miRNA and the mRNA 3-UTR region by providing an environment that tends to stabilize the miRNA/mRNA complex. A key player in the protein scaffold of the RISC is the Argonaute protein family. These proteins represent the catalytic part of the RISC. Indeed, they possess endonuclease activity that may be important in mRNA destruction, as well as in the activation of a precursor miRNA to the active, mature form. Interestingly, it has been established that the structure of the Argonaute protein contains a highly conserved basic pocket that can bind to the 5'-region of the miRNA. The fact that parts of the Argonaute proteins are strongly conserved throughout living forms and found even in bacteria points to their role having developed early in the history of evolution.

We have seen data in previous chapters suggesting that miRNAs may be surrounded by much more developed three-dimensional protein/protein cross-linked cages than other DNA or RNA molecules in the cells in a given formalin-fixed, paraffin-embedded tissue. This may partly explain why, in general, microRNAs tend to need stronger pretreatment conditions than do DNA or RNA molecules in the tissue of interest. The information just discussed relative to the RISC proteins in general and the Argonaute proteins in particular provides us with a more solid biochemical basis to speculate that miRNAs may be surrounded by dense, large protein coats that would, of course, have a strong influence on the ability to detect these very small miRNAs by in situ hybridization.

As indicated, miRNA research has fueled enormous interest in coexpression analyses. There are several ways you can determine whether a given miRNA may be inhibiting a specific mRNA:

1. Do a computer-based scan that matches the so-called seed region of the miRNA with the complementary sequence in the 3-UTR of the mRNA of interest. This can be done via some websites such as TargetScan.
2. Use the luciferase assay and, by making vectors that include the luciferase molecule downstream of the 3-UTR sequence, document that the miRNA can inhibit the expression of luciferase, which would happen if the upstream mRNA was not being transcribed due to the presence of the miRNA.
3. Show that by mutational analysis to the 3-UTR region noted in point 2, earlier in the chapter, that the inhibition of expression of the mRNA via the miRNA is lost. That is, you can change the sequence of the 3-UTR such that the miRNA is no longer able to bind to it. Thus, luciferase is normally expressed.

Although each of the three methods detailed here is useful, all are done in the test tube and, thus, none directly examine the physiologic effect of the miRNA on the putative mRNA target. This can only be done with coexpression analysis for the miRNA and target in question. Coexpression analyses also allow us to readily answer a question that cannot be answered if the miRNA analysis is done by solution-phase methods, such as qRTPCR, since the tissue is ground up and destroyed in such analysis. Specifically, the coexpression analysis of the microRNA and phenotypic marker will allow us to determine the specific phenotype of the cell that is expressing the miRNA. These two key points are illustrated in Figs. 11.1 and 11.2. In Fig. 11.1, panel A shows a strong expression of a microRNA in the spinal cord of a mouse that was paralyzed after induced ischemia to the spinal cord. When compared to the control, this microRNA was much increased in the mouse with the ischemic cord. Note the distribution of the microRNA; it is present in the ventral and dorsal horns of the spinal cord. This is the exact region that the larger motor neurons are present in the spinal cord. Of course, the large motor neurons would be the cells that are directly responsible for paralysis. Indeed, these are the target cells of the poliovirus, which, of course, causes paralysis in its victims. To further document that these cells that are positive for the miRNA upregulated in induced spinal cord paralysis in the mouse are neurons, immunohistochemistry analysis was done on the serial section for the neuron-specific protein Fox 3. As you recall, the use of serial sections allows us to study the same cell populations. Note that Fox 3 has the same distribution as the miRNA (panel B). To get further verification that the miRNA was expressing cells, the sections were treated with the miRNA first and then the protein Fox 3. These data are presented in panel C. The miRNA is blue, and the Fox 3 protein is brown.

When these coexpression images were analyzed by the Nuance system, the miRNA signal was converted to a fluorescent blue and the Fox 3 signal to a fluorescent red. Cells with both targets are evident as fluorescent yellow. As is evident from Fig. 11.1, panel D, many cells that express the miRNA are neurons, as they coexpress the neuronal marker Fox 3.

Figs. 11.2 and 11.3 show two different examples of how coexpression analysis can be very useful for the study of putative targets of microRNAs. Work done by Dr. Michela Garofalo in the laboratory of Dr. Carlo Croce showed that, in lung cancers, certain critical oncoproteins may be directly regulated by specific microRNAs. Two such oncoproteins that are increased in lung cancer, and associated with a worsened prognosis, are cMET and Src. These researchers had strong data that indicated miR-203 downregulation could be responsible, in large part, for the increased expression of cMET and Src in lung cancers. Clearly, this would give clinicians another possible route to treat lung cancers, because the introduction of miR-203...
would simultaneously reduce the expression of these two (and probably other) oncoproteins. When we did serial section analysis of miR-203, cMET, and Src in the lung cancers, it was obvious that some cancer cells were expressing each of the three targets. The best way to examine their physiologic interdependence was to do coexpression analysis. As seen in Figs. 11.2 and 11.3, the cancer cells that are expressing miR-203 are clearly not expressing cMET or Src, and vice versa. This gives a powerful additional layer to the foundation of the hypotheses that miR-203 may be regulating these two important oncoproteins.

Let’s examine Figs. 11.4 and 11.5 for the “reverse image” of the downregulation experiments seen in Figs. 11.2 and 11.3. Fig. 11.4 shows a series of cancer cell lines that were either exposed to the oncolytic virus called reovirus or given a “sham” infection. It is known that reovirus can kill cancer cells and that its entry into malignant cells is associated with proteins such as caspase-3 (which can lead to the direct apoptosis of the cancer cells) and p38 (which enhances reoviral growth probably reflects its ability to inactivate PKR). Note, in panel A, we did coexpression of reovirus and caspase-3 in a reovirus-treated cell line. Of course, you cannot examine serial sections of cell preparations, because you simply add the...
FIGURE 11.2  Coexpression analyses of microRNA-203 and cMET in lung cancer. There are times, especially with microRNA and its putative target, when serial section analysis of the two targets show that in a given nest of cancer cells some of the cancer cells are making the microRNA and some of the cancer cells are making the putative target. In such cases, serial section analyses cannot tell us if these are two distinct populations of cells. To determine whether the cancer cells that express the miRNA are expressing or not expressing the putative protein target, we must do coexpression analyses. The routine microscopy analysis (panel D) shows a nest of cancer cells in which the blue of the tumor suppressor microRNA-203 and the red of the oncoprotein cMET are intermingled. The Nuance system converted the microRNA signal to fluorescent blue (panel A) and the cMET signal to fluorescent red (panel B). Merging the two images (panel C) shows that miR-203 positive cells do not express cMET, and vice versa. This bit of data supports the large amount of solution-phase data that suggests that the miR-203 can regulate cMET expression.

FIGURE 11.3  Coexpression analyses of microRNA-203 and the oncoprotein Src in lung cancer. In this tumor the slide was analyzed for miR-203 and Src after the optimal conditions were detected for each target in this particular tissue. It is clear that the tumor cells in this area do not express miR-203 (panel A, fluorescent blue), but that basically all express Src (panel B, fluorescent red). Thus, coexpression analyses (panel C) showed no tumor cells expressing both. This figure also shows the value of adding a counterstain (methylene green), because it allows us to see the cancer cells (lower part of image) and adjacent stromal cells (upper part of image, panel D). Still, I prefer to use the regular RGB image (panel D, Fig. 11.2) to show the histologic features of the tissue when using the Nuance system.
cells directly to the silane-coated slide. (Of course, you could put the cells in a paraffin block and then cut sections, but I do not see any advantage to adding this step.) Again, it is simply not possible for our eyes to discriminate against the red reovirus signal from the brown caspase-3 signal from the blue hematoxylin signal. But, of course, a computer-based system can do this easily. First, note in panel B that the different colors can be easily separated by the Nuance system (caspase is fluorescent green, and the insert shows the fluorescent red signal of the reovirus). When the signals are mixed, you can see the fluorescent yellow that indicates most of the cancer cells with the reoviral protein also express caspase-3. As a negative control, reovirus and PKR were analyzed by coexpression (panel D). Note the lack of coexpression, which serves as a good negative control of the reovirus/caspase-3 data. Panel E shows that the reovirus positive cells also express p38 in the reovirus-infected cell line. Panel F is the corresponding negative control of reoviral/p38 coexpression analyses in a cell line not exposed to the virus. These images show the value of computer-based analyses when doing coexpression analyses.

Fig. 11.5 shows the equivalent data in reoviral-infected tissue. The tissue came from a patient with colon cancer who had liver metastasis. The patient was given reovirus before the removal of the liver metastasis. We did coexpression of reovirus with tubulin, p38, and...
caspase-3 in serial sections. The purpose of showing the data is threefold: (1) it highlights the value of analyzing serial sections for coexpression analyses, as we are examining the same sets of cells; (2) it shows that we cannot determine with regular microscopy whether cells are coexpressing two different proteins if the two are in the same cellular compartment (each protein in this set is cytoplasmic based); (3) we can see perhaps the strongest feature of in situ hybridization and immunohistochemistry: the ability to document that the reovirus is specifically targeting cancer cells. Such information could not be obtained from PCR or Western blot-based data.

Reovirus needs to attach to the microtubulin scaffold when productively infecting a cell. As seen in Fig. 11.5, panels A (regular image) and definitively in panel B (Nuance-based image), most of the reovirus-infected cells indeed coexpress microtubulin. Thus, the virus has not been simply passively absorbed by the cells but, rather, is productively replicating in the cancer cells. The serial section shows that the reovirus positive cells coexpress p38 (panel C), and that a smaller percentage of these cancer cells are also expressing the protein caspase-3 that likely signals their death via apoptosis (panel D).

Now let’s look at different ways we can do coexpression analyses.

**Different methodologies for coexpression analyses**

Use either two probes or two antibodies that can detect their respective targets in completely distinct cell populations

Using either two probes or two antibodies is certainly the easiest way to do coexpression analyses. The only two
criteria for using this simple form of coexpression analyses are listed:

1. The two different targets must be in two morphologically distinct and completely different cell populations.
2. The two different targets need to have similar immunohistochemistry or in situ hybridization optimization profiles.

In this book, we have already seen many examples in which two different protein epitopes were detected simultaneously by doing immunohistochemistry with one chromogen and two separate primary antibodies. Fig. 11.6 shows one such example. The tissue is a cervical biopsy from a woman who had cervical intraepithelial neoplasia (CIN). This is the precursor lesion to cervical cancer, and it is invariably associated with human papillomavirus (HPV) infection. The lesion was analyzed at the same time with cytokeratin (epithelial marker), MCM2 (mitotic activity marker), and CD45 (lymphocyte marker). Of course, we can easily differentiate the cytoplasmic signal of cytokeratin from that of CD45. The reason is that the cytokeratin is in the very large, stacked squamous cells, whereas the CD45 signal is in the much smaller lymphocytes that dominate in the submucosa. The MCM2 signal is easily differentiated from the other two signals because it is nuclear and the other two are cytoplasmic.

![Fig. 11.6 Coexpression analysis with one chromogen: CD45; keratin; and MCM2. The simplest type of coexpression analysis is when two or more targets are present in different cell types and/or cell compartments that are easily differentiated on cytologic grounds. Keratin is found in the cytoplasm of squamous cells, MCM2 is a nuclear epitope present in rapidly dividing cells, and CD45 is present in the cytoplasm of lymphocytes. Thus, we can analyze a CIN biopsy for all three targets and get the same exact results as if three serial sections were used, one for each target. This saves reagents and time, and reminds us that the generation of a given signal with immunohistochemistry or in situ hybridization will not interfere with the simultaneous development of another signal. Note in panel A, and at higher magnifications in panels B and C, that the entire squamous epithelia clearly shows the cytoplasmic signal that corresponds to cytokeratin AE1/3, whereas the more basal cells of the squamous cell layer show the intense nuclear signal of MCM2. Many cells in the stroma show the cytoplasmic signal of CD45; T- and B-cells are invariably present in the stroma of the cervix. These three distinct regions/cellular localization patterns are seen in panel D as small arrow (squamous cells), middle-sized arrow (MCM2), and large arrow (lymphocytes).](image-url)
Note in Fig. 11.6, panel A, that the entire squamous epithelia clearly shows the cytoplasmic signal that corresponds to cytokeratin AE1/3, whereas the more basal cells of the squamous cell layer show the intense nuclear signal of MCM2. Finally, the stroma, as expected, shows the CD45 positive cells because the lymphocytes will predominate in this area. These three distinct regions/cellular localization patterns are seen in panel D as small arrow (squamous cells), middle-sized arrow (MCM2), and large arrow (lymphocytes).

As you are probably aware, several companies offer excellent colabeling kits. These kits allow you to detect two (or more) antigens in a given immunohistochemical experiment. These commercial kits use one color for one antigen and another color for the other antigen, which typically are located in completely different cell populations. The results I have seen from such products are excellent. However, we can do the same experiments right now using the same chromogen under the guidelines outlined previously. I see three solid advantages to this simplest of coexpression analysis:

1. It strengthens our surgical pathology/histopathology knowledge because it requires us to be able to differentiate different cell types and the cytoplasm from the nucleus.
2. It strengthens our immunohistochemical and in situ hybridization knowledge because it requires us to have a thorough knowledge of the optimization profiles of the two or more targets.
3. It allows us to save money on reagents and generate much more data with fewer slides and experiments. I suppose this may reflect my Vermont upbringing, as Vermonters are known for their frugality!

Let’s look at one more example of doing multiple analyses (again for three distinct targets) at the same time, using the same chromogen. The tissue is breast cancer. The three targets are as follows: (1) the cancer cells (of course, easily differentiated by the disorganized growth pattern and the variation in nuclear size, shape, and color. Do you remember the name of the classic pattern of adenocarcinoma where glands form within the larger gland? See below); (2) smooth muscle cells (easily distinguished since they line large blood vessels); and (3) lymphocytes (small round cells in the stroma).

As seen in Fig. 11.7, panel A, the cancer cells are positive for AKT (nuclear-based signal after this oncogene is activated—small arrow), the lymphocytes are positive for CD45 (intermediate arrow), and the smooth muscle cells are positive for SMA (large arrow). Panel B shows the adenocarcinoma and lymphocytes at higher magnification, and panel C shows the smooth muscle cells at higher magnification. By the way, this tissue was 12 years old, and the AKT signal was not even evident in the original material. However, when the tissue was pretreated with IHCPerfect, the AKT signal became evident (panels A and B), and the CD45 and SMA signals became stronger (panel C, after rescue of the signal, and panel D, the original data without pretreatment). These data remind us that making a stronger three-dimensional macromolecule cross-linked network, of course, not only makes invisible signals evident but can also make signals that are 1 + or 2 + become stronger. By the way, the adenocarcinoma pattern in panel A is called the cribriform pattern.

Of course, there will be many times when we want to analyze two or more targets in the same tissue sections in cases where the different targets will be present in either the same cell types and/or the same subcellular compartments. In such circumstances, we will need to use other strategies, which we discuss next.

**Analyze the same section more than once**

Let’s go over two points by analyzing the same tissue section two or more times with immunohistochemistry, in situ hybridization, or both:

- **Point 1.** Although serial section analysis is very helpful with coexpression analyses, it cannot reliably tell us whether a given cell is making each target.
- **Point 2.** Under a regular light microscope, you cannot reliably differentiate two different colored signals if they are in the same cellular compartment of the same cell.

Let’s discuss each point in turn.

Regarding point 1, let’s examine Fig. 11.8, panels A and B. Note that these are serial sections, with panel A showing the histologic distribution of cKIT (CD 117) in the tonsil and panel B showing the distribution of IL22 in the serial section. Note the cluster of adipose cells on the right of each image and the solitary fat cell on the left. Clearly, these two targets show a similar distribution pattern in the interfollicular region of this tonsil. But we cannot reliably say whether a given cell has both targets, even though the sections are only 4 μm apart. Compare this to Fig. 11.1. Here, we could clearly say that the miRNA and the protein target Fox 3 had the same population distribution (larger motor neurons of the spinal cord), which is strong evidence that this cell type is expressing both. But in the tonsil, the cells are smaller and not as cytologically distinct. So, the best plan of action with the tonsil is to go to computer-based coexpression analysis. The results of such an experiment are seen in Fig. 11.8, panel C (light microscopy), where the same tissue was analyzed using Fast Red for cKIT and then using DAB for IL22. The Nuance system converted the cKIT image to fluorescent red (panel D) and the IL22 signal to
fluorescent green (panel E). By merging the images, the Nuance system clearly shows that cells making cKIT (a marker of natural killer cells) also express IL22 (seen as fluorescent yellow in panel F). Indeed, all the cKIT cells are also IL22 in this image.

It follows from such data that a computer-based coexpression system will be very helpful when doing coexpression analyses if both targets are in the same cell types and in the same subcellular compartments. I want to stress that the analysis of serial sections of the different targets is still an essential part of the coexpression analyses in such cases.

Now let’s review some observations that are relevant to coexpression analyses that we have discussed in previous chapters before we go on to point 2:

A. You can still get good immunohistochemical or in situ hybridization results even if the tissue has been stained with hematoxylin, eosin, or hematoxylin and eosin.

B. You can still get good immunohistochemical or in situ hybridization results even if the tissue has already been analyzed by immunohistochemical or in situ hybridization.

C. Point 2 assumes one of two conditions:
   a. The second target has the same pretreatment optimizing regime as the first target. (This is by far the most common scenario where, for example, each target needs either protease digestion or antigen retrieval/DNA/RNA retrieval for optimal detection.)
   b. The pretreatment of the first target does not interfere with the pretreatment regime of the second target. (A good example of this is where no pretreatment is needed to detect HPV in situ followed by immunohistochemical for a protein that needs antigen retrieval for optimal detection.)
D. It is *not* possible to reliably remove the precipitate when using DAB, NBT/BCIP, or Fast Red (Ventana) as the chromogen.

E. It *is* possible, however, to use a chromogen that is soluble in ethanol or xylene such that you can do immunohistochemistry, photograph the results, and then remove the chromogen and stain the same tissue for another target using a different colored chromogen.

Points A–C remind us that we can readily stain a given tissue for two or more targets under the conditions listed in these points, and then use a computer-based system to tabulate and analyze our data. Point E reminds us that we do not have to do this if we use a chromogen (such as Fast Red from Biogenex) that is ethanol or xylene soluble.

Regarding point 2—not being able to reliably differentiate two different colored signals if they are in the same cellular compartment of the same cell under a regular light microscope—this is evident by reexamining panel D of Fig. 11.8. These points remind us that, if two or more signals are present in the same cells in the same cellular compartments, then we need to follow one of two protocols:

- Protocol 1. Do the first test with Fast Red as the chromogen (soluble in ethanol), aquamount the slide,
photograph the regions of interest, remove the coverslip, remove the chromogen with an ethanol wash, then do the second immunohistochemical reaction, and photograph the same cells.

- Protocol 2. Do the two separate immunohistochemical and/or in situ hybridization reactions with two separate chromogens. Then analyze with a computer-based system that is capable of isolating the different chromogens, mixing them, and thus performing the coexpression analyses.

Having done many experiments using Protocol 1, I can attest that it is not too difficult. Of course, it is time consuming. Protocol 2 is much quicker, and you have a permanent record of both target visualizations. Of course, Protocol 2 requires investment in a computer-based coexpression analysis system (see Appendix).

Before we leave this section, I would like to go over the use of coexpression analyses as a quality control tool for immunohistochemistry. Fig. 11.9 shows the results for immunohistochemistry using CD68 as the chromogen in lung cancer. Very few cells were positive (panel A shows the results after Nuance conversion of the DAB image to fluorescent green). The suspicion was that the CD68 DAB signal was not optimized. To confirm this, we did CD68 immunohistochemistry again, but this time including protease digestion and using Fast Red as the chromogen. Panel B shows many more positive cells (regular light microscopy images). Panel C shows that by then doing coexpression for CD68 (DAB and Fast Red) and using fluorescent yellow as the coexpression indicator, the CD68 assay for this tissue needed the protease digestion step. Panel D shows the same image, but the fluorescent blue of the hematoxylin counterstain was added to better appreciate the tissue morphology.

**FIGURE 11.9** Coexpression analysis for the same antigen as a quality control tool. Panel A shows the immunohistochemistry using CD68 as the chromogen in a lung cancer. Note that very few cells were positive (panel A shows the results after Nuance conversion of the DAB image to fluorescent green). The suspicion was that the CD68 DAB signal was not optimized, as the laboratory used too low a concentration of the primary antibody and the incorrect pretreatment. To confirm this, we did CD68 immunohistochemistry again, but this time including protease digestion and using Fast Red as the chromogen. Panel B shows many more positive cells (regular light microscopy image). Panel C shows that by then doing coexpression for CD68 (DAB and Fast Red) and using fluorescent yellow as the coexpression indicator, the protease digestion and higher concentration of the antibody produced many more positive cells (fluorescent red alone). This again reminds us that a prior immunohistochemistry reaction should not interfere with a subsequent reaction, unless the pretreatment for the first antigen eliminates the possibility of detecting the second antigen. Panel D shows the same image, but the fluorescent blue of the hematoxylin counterstain was added to show the nuclei of the cells.
Use computer-based coexpression

The rest of this chapter is devoted to doing coexpression analyses of two or three targets using a computer-based coexpression analysis system.

Let me start by presenting the protocol I recommend for coexpression analysis where it is assumed that the two (or more) targets in question are located, at least in part, in the same subcellular compartment of the same cells. I begin by assuming that a large percentage of the readers interested in doing coexpression analyses wish to do so for a microRNA and a putative target. Thus, the protocols use microRNA by in situ hybridization and putative protein target by immunohistochemistry as the template system. If you are interested in doing protein/protein coexpression, then simply substitute the word “protein” for the word “microRNA.” If you wish to do RNA/DNA in situ hybridization coexpression, just substitute the letters “RNA” for “microRNA” and the letters “DNA” for “protein,” and so on.

Protocol for coexpression analysis in formalin-fixed, paraffin-embedded tissues

Step one: Optimizing step

Determine the optimal conditions, including necessary pretreatment (if any) and probe concentration for the microRNA of interest using the protocol in Chapter 7, Recent Improvements in Immunohistochemistry and In Situ Hybridization. Simultaneously, determine the optimal conditions including necessary pretreatment conditions (most likely some pretreatment will be needed) and primary antibody concentration for the epitope of interest using the protocol in Chapter 8, The Basics of Histologic Interpretations of Tissues. Remember to test both the microRNA and protein with no pretreatment, protease alone, “RNA or antigen retrieval” alone, and RNA/antigen retrieval plus protease digestion, because this will be important information.

Step two: Determine the order of coexpression analysis

Write down the results for the optimizing experiments for the tissue of interest for the detection of the microRNA and the protein. Remember to pay close attention to the results of all experimental conditions. If antigen retrieval yielded a 3+ signal for immunohistochemistry, but protease digestion alone and antigen retrieval + protease digestion each yielded a 2+ signal with no background, the latter two conditions may be acceptable for coexpression analyses. This is especially true if using the Nuance-based computer system for analyses because it is much more sensitive to the signal than are our naked eyes.

Choose one of the following sets of conditions:

A. miRNA—no pretreatment optimal and protein, protease digestion or antigen retrieval optimal
   The microRNA in situ hybridization must go first (recall, this is very rare).

B. miRNA—protease digestion optimal and protein, protease digestion optimal
   Either the microRNA in situ hybridization or protein immunohistochemistry can go first. If the latter is done first, remember to follow RNase precautions for the microRNA in situ hybridization.

C. miRNA—protease digestion optimal and protein, antigen retrieval optimal
   This is a common situation. Under such circumstances, examine the scores for the microRNA in situ hybridization if RNA retrieval was used and the scores for immunohistochemistry if protease digestion alone was used. Remember that antigen retrieval alone (or protease digestion alone) may yield too much background for immunohistochemistry with one concentration, but each may yield excellent results for both if the concentration of the primary antibody is reduced. In my experience, in more than 90% of these cases (miRNA—protease digestion is optimal, and protein—antigen retrieval is optimal), you can find conditions that will allow both the miRNA and protein to be detected simultaneously.

D. miRNA—RNA retrieval optimal and protein, antigen retrieval optimal
   This too is a common situation. In this case, I recommend doing the microRNA in situ hybridization first and then the protein detection by immunohistochemistry. Remember to not redo the antigen retrieval for the protein or the signal will probably be diminished.

E. miRNA—RNA retrieval optimal and protein, protease digestion optimal
   This is an unusual situation. When it does occur, in my experience, the protein can usually be detected satisfactorily with antigen retrieval if the primary antibody concentration is adjusted.

To restate this important point, in most cases scenario C or D here will apply. This is another reason I have stressed in the optimization protocols for microRNA/RNA and DNA in situ hybridization and protein detection by immunohistochemistry that you test different concentrations and different pretreatment conditions (nothing, protease digestion, antigen retrieval, and antigen retrieval + protease digestion) because this will allow you to see the range of possible pretreatments.
for coexpression analyses. Finally, I purposely left out some possibilities, such as both microRNA and protein need no pretreatment, because I have never seen this and, if it happened, clearly you could test each in sequence.

**Step three: Test serial sections for the microRNA by in situ hybridization and the protein by immunohistochemistry**

I consider Step three to be the key step when doing coexpression analyses. In this step, you take serial sections and analyze them for the microRNA and protein of interest. You then carefully record and interpret the data.

In this step, you bring together the two critical fields of expertise needed for optimal interpretation of immunohistochemistry and in situ hybridization. These are, of course, an in-depth knowledge of molecular pathology and histopathology. I like to photodocument the data at this stage, taking photos of the same areas of the serial sections to study the same groups of cells for the two different targets.

The reason I consider Step three to be the most important step of coexpression analyses is that its goal is to let you determine the interplay between the two (or more)

![Figure 11.10](image-url)

**FIGURE 11.10** Use of multiple coexpression analyses to dissect the molecular “cross-talk” between cancer and inflammatory cells in lung cancer: Part A: miR-29a was much increased in the cancer cells (upper and mid part of panel A), but not in the cells of the adjacent normal lung present at the tumor interface. In the serial sections of this lung cancer, CD9 was found in the cancer cells and in some inflammatory cells, but only at the interface of the cancer (panel B; CD9 is a marker of exosomes, which are the vehicle that cells can use to transport molecules to other cells). A higher magnification of the tumor interface shows that the CD9 positive cells had the cyto logic features of macrophages (panel C), reminding us again of how invaluable a strong foundation in histopathology can be in such interpretations. Analysis of the serial section for TLR-8 shows that the positive cells were found mostly at the tumor interface and in cells with the cytologic features of macrophages (panel D and, at higher magnification, panel E). Using the fact that macrophages routinely traffic from the cancer to the regional lymphoid accumulations/lymph nodes, we obtained additional evidence that the CD9 positive cells were probably macrophages, as they were concentrating in the lymphoid aggregates around the tumor (panel F).
FIGURE 11.11 Use of multiple coexpression analyses to dissect the molecular “cross-talk” between cancer and inflammatory cells in lung cancer:

Part B: In these experiments, the cancer cells were transfected with anti-miRNAs, injected into the mouse, and then the lung metastases were examined to determine whether the anti-miRNA had rendered the cancer cells incapable of metastasis. These experiments are based on the observation that not all cancer cells transfected with a given anti-miRNA will acquire the anti-miRNA. Indeed, we did document that the cancer cells that metastasized did not have the sequence of the “anti-miRNA” used to silence the oncomiRNA (panel A), but did still have the sequence of the oncomiRNA (panel B). We then showed that the metastases in the mice showed the same geographic pattern of the miRNA and CD9 (concentrating at the tumor interface; panel C) as we noted in the human tumor (Fig. 11.10). The coexpression analyses did indeed show that the miRNA and CD9 were concentrating in a thin rim specifically at the tumor interface (panel D), but not in the alveolar macrophages 1 mm away in the normal lung (panel E). The coexpression analyses also showed that some of the miRNA+ cells at the tumor interface were CD68+ macrophages (panel F) which, since they were not making the miRNA, must have acquired it via the exosomal release from the cancer cell, in a strong example of how cancer cells can influence the surrounding benign inflammatory cells via “cross-talk” mediated by the microRNAs.
targets before you do the computer-based analyses. In other words, I prefer to use the computer-based analysis system as the corroborative test and not the primary test for coexpression analysis.

Let’s look at a couple of examples in Figs. 11.10 and 11.11. This work came from the laboratory of Dr. Carlo Croce and included several of his team members, including Dr. Muller Fabbri and Federica Calore. They had extensive solution-phase evidence suggesting that certain microRNAs could be exported by cancer cells and, in turn, modulate inflammatory cell function. They provided me with extensive mouse tissue and human tissue with a lung cancer model. They wanted me to address the issue of whether coexpression analysis could provide additional evidence that there was direct trafficking between cancer and inflammatory cells. Some representative data are shown in Fig. 11.10. Let’s summarize what this figure shows:

1. In the human cancer, the microRNA was found mostly in cancer cells (panel A).
2. In the serial sections of the human cancer, CD9 was found in the cancer cells and in some inflammatory cells, but only at the interface of the cancer and the benign tissue (panel B; CD9 is a marker of exosomes, which are the vehicle that cells can use to transport molecules to other cells).
3. A higher magnification of the tumor interface shows that the CD9 positive cells had the cytologic features of macrophages (panel C).
4. Analysis of the serial section for TLR-8 shows that the positive cells were found mostly at the tumor interface and in cells with the cytologic features of macrophages (panel D and, at higher magnification, panel E).
5. As macrophages routinely traffic from the cancer to the regional lymphoid accumulations/lymph nodes, we looked to see whether the CD9+ cells were found in the regional lymphoid accumulations. As you can see in panel F, CD9+ cells were indeed concentrating in these areas.

So, to summarize, before doing any coexpression experiments, we obtained a lot of information from the serial section experiments for the different targets of interest. It seems clear that the miRNA is associated with an exosomal marker, but in a very specific geographic locale—specifically, where the tumor meets the adjacent benign tissue, commonly called the tumor interface. It is also clear that the tumor interface is the place where macrophages that contain the exosomal marker CD9 are congregating. Finally, the TLR-8 accumulation at this site would be consistent with evidence that the cancer cells may indeed be modulating the macrophage’s behavior.

Dr. Croce’s laboratory staff then documented that if they partially knocked out the miRNA in the mouse model, then far fewer lung metastases were evident. Of course, partial knockout via transfection will allow some cancer cells to still express the microRNA. Here is a good example of the power of in situ hybridization. We analyzed the lung metastases in the mice with the partial knockout of the miRNA. Our hypothesis was that these tumors would still express the miRNA, and not the “anti-miRNA.” As seen in Fig. 11.11, this indeed was the case. The cancer cells did not have the sequence of the “anti-miRNA” used to silence the oncomiRNA (panel A), but did still have the sequence of the oncomiRNA (panel B). We when showed that the metastases in the mice showed the same geographic pattern of the miRNA and CD9 (concentrating at the tumor interface; panel C). Similar patterns were seen with the macrophage mouse marker and TLR-7 (data not shown).

It was only after having all of this data that we did the coexpression analyses. These analyses did indeed show that the miRNA and CD9 were concentrating in a thin rim specifically at the tumor interface (panel D), but not in the alveolar macrophages 1 mm away in the normal lung (panel E). The coexpression analyses also showed that some of the miRNA+ cells at the tumor interface were CD68+ macrophages (panel F), which, since they were not making the miRNA, must have acquired it via the exosomal release from the cancer cell. The final point to make about Figs. 11.10 and 11.11 is that they show that the different targets of interest are being expressed in the same subcellular compartment (primarily, the cytoplasm), and that the cells expressing these different targets overlap (cancer cells and inflammatory cells). This is why we needed to do the coexpression analyses. If the targets were in the same cell populations but different subcellular compartments, there may not be a need to do computer-based coexpression analysis, but, rather, we could rely on the serial section and colorimetric coexpression data.

The point of showing Figs. 11.10 and 11.11 is not to review the data of cancer cell and macrophage cross-talk, although it is, to put it mildly, fascinating. It is, however, to show that our molecular pathology and surgical pathology expertise can give us a great deal of information with serial section analyses before we do the coexpression analysis.

**Step four: Do at least two different optimizing protocols when doing the coexpression analysis**

The final important technical point makes perfect sense considering what we have learned about optimizing signals for in situ hybridization and immunohistochemistry.
Let’s summarize the main points here, as they are the basis of Step four:

1. For in situ hybridization, signal and background are dependent on the probe concentration and the pretreatment conditions.
2. For immunohistochemistry, signal and background are also dependent on the primary antibody concentration and the pretreatment conditions.
3. The optimal conditions for two different targets, even if in the same subcellular compartment, can differ greatly in a given tissue.
4. The optimal conditions for a given DNA, RNA, or protein target can vary from tissue to tissue depending on fixation conditions, over which we usually have no control and have no specific knowledge about.

To get right to the point, the preceding four points are going to make our coexpression analyses more challenging from a technical standpoint! Even if we have optimized a microRNA by in situ hybridization and its putative protein target using immunohistochemistry on a given tissue, and found that each case protease digestion was optimal at a specific concentration, it does not mean that we can be certain that the same optimal conditions may apply to a different tissue. I also want to stress that this problem seems to be more important for microRNA in situ hybridization. As we discussed at length in previous chapters, the optimal conditions for HPV in situ hybridization (using this as a model of DNA in situ hybridization) are usually rather straightforward. You typically need to do either no pretreatment or protease digestion to optimize the HPV in situ hybridization for most tissues. Yes, in some tissues, DNA retrieval with or without protease digestion is needed for successful HPV in situ hybridization, but these are the exception, not the rule. Not so with microRNA in situ hybridization. You may recall that we theorized that this is the case because microRNAs are typically associated with a much denser, more complex three-dimensional protein/protein cross-linked network than other DNA or RNA sequences. Whatever the explanation, if you are doing coexpression analyses with microRNA in situ hybridization and your putative target, you will face the challenge of needing to reoptimize the optimal conditions for each additional tissue.

It follows that if your interest is primarily coexpression with two distinct proteins using immunohistochemistry, then Step four will not be as relevant for you. In most cases, the optimal conditions for a given protein for immunohistochemistry are relatively constant and, as we have stressed throughout this book, in most cases they will involve antigen retrieval. This is why coexpression with two or more proteins that exist in separate cell compartments and/or different cell types allows for such simple coexpression analysis. You need only to add the two (or more) primary antibodies, do the reaction with one chromogen, and be very confident that the reaction will work very well because you have already determined that each primary antibody requires the same pretreatment conditions.

But, since I am assuming that many of you who want to do coexpression are interested in examining a microRNA by in situ hybridization and its putative protein by immunohistochemistry, let’s discuss how to address the previous four points in a protocol. Here is what I recommend:

**Extended protocol for microRNA in situ hybridization and putative protein target coexpression analyses**

1. Have your technician place serial sections on 10 unstained silane-coated slides, labeling the slides 1–10. As always, make certain that there are from two to three sections per slide, depending on the size of the formalin-fixed, paraffin-embedded tissue.
2. Use slides 1 and 2 to determine the optimal conditions for microRNA in situ hybridization on this tissue by comparing the following variables:
   - No pretreatment and protease digestion (slide 1)
   - RNA retrieval and RNA retrieval + protease digestion (slide 2)
   (Use the same concentration of the microRNA probe that you determined was optimal when you initially optimized the microRNA in situ hybridization; see Step three of Chapter 7: Recent Improvements in Immunohistochemistry and In Situ Hybridization)
3. Use slides 3 and 4 to determine the optimal conditions for putative protein target by immunohistochemistry on this tissue by comparing the following variables:
   - No pretreatment and protease digestion (slide 3)
   - Antigen retrieval and antigen retrieval + protease digestion (slide 4)
   (Use the same concentration of the primary antibody that you determined was optimal when you initially optimized the epitope detection; see Step three of Chapter 8: Recent Improvements in Immunohistochemistry and In Situ Hybridization)
4. Do not counterstain slides 1–4. Rather, coverslip them and then photograph the sections that represent the specific condition that was optimal for the detection of the microRNA and the specific condition that was optimal for the detection of the putative protein target.
5. After photographing the data, write down your observations as to which specific cell type or types are expressing the microRNA of interest and what specific cell type or types are expressing the putative protein target of interest. Also note what specific cellular compartments contain the microRNA and protein of interest.

6. Use slide 5 to perform the microRNA in situ hybridization reaction using the optimal conditions you documented above in step 4 of this protocol. However, since you have at least two sections per slide, use one section as the negative control (either omission of the probe or use of a scrambled or irrelevant probe).

7. Similarly, use slide 6 to perform the immunohistochemistry reaction using the optimal conditions that you documented in step 4 of this protocol. However, since you have at least two sections per slide, use one section as the negative control (typically omission of the primary antibody).

8. Confirm under the microscope that slides 5 and 6 performed as expected.

9. Use slide 5 to do the immunohistochemistry reaction for the putative protein target of the microRNA.

10. Use slide 6 to do the in situ hybridization for the microRNA of interest.

   (Of course, the order may be dictated by the optimal conditions for the microRNA and protein target detection; see “Step two: determine the order of coexpression analysis” earlier in this chapter.)

11. If you are using NBT/BCIP as the microRNA counterstain and DAB as the chromogen for the putative protein target, you can use Nuclear Fast Red as the counterstain. Do not use hematoxylin for a counterstain with NBT/BCIP as the chromogen. For the Nuance coexpression analyses, you do not need a counterstain. However, you do need a counterstain if you are using the InForm quantification system for coexpression analysis. If you are using NBT/BCIP and either Fast Red or DAB, methylene green can serve as an effective counterstain.

   Let’s look at an example of the preceding protocol. Before we do, there is one more point to make. Assuming that you are using a computer-based system to document the coexpression data, it is important to realize that the computer is doing the equivalent of a three-dimensional “CT” scan of the cell. But instead of measuring differences in density of tissue, it is measuring differences in the color staining of the cell. Also, the computer will not discriminate between cytoplasm and nucleus when doing this analysis. Rather, it will use a plane of about 150 nm, and if the signal is present in the nucleus and the other signal is present directly above the nucleus in the cytoplasm (that is, in the same plane), then the computer will score the cell as “positive for coexpression.” So this is not the same as colocalization in which only two signals present in the exact same subcellular compartment are scored as positive. Rather, this is truly “cellular coexpression analysis” in which we are trying to determine whether a given cell does or does not have our two targets of interest. Of course, that is what we are interested in with such experiments, and not true “colocalization.”

   Let’s look at Fig. 11.12 to illustrate this important point. The RNA that was detected is EBER-1 and 2. This is an RNA made in abundance by the EBV, which is also called human herpesvirus 4 (HHV-4), and it is nuclear based. The same tissue was also analyzed for the EBV protein bZlf-1 by immunohistochemistry with DAB as the chromogen (panel A shows the coexpression as seen under light microscopy, and panel C shows the Nuance-isolated EBER-1 and -2 signal). As is evident, the bZlf-1 protein is also nuclear based (panel C, shown as a fluorescent red signal by the Nuance system). If cells infected with HHV-4 show both EBER-1 and -2 as well as bZlf-1, then this indicates that the infection is lytic as opposed to latent. It is not possible to determine if the EBER-1 and -2 RNA positive cells also contain bZlf-1 protein, although it is certainly clear many cells are expressing at least one of these targets. The Nuance system (Fig. 11.12, panel D) clearly shows this is a lytic viral infection, because many of the cells show the fluorescent yellow color indicative of coexpression.

   Now let’s go to Fig. 11.13. These series of experiments were done in collaboration with Dr. Croce’s laboratory and were headed by Dr. Michela Garofalo. She has done extensive and excellent work on the microRNA modulators of various cancers, including lung. In this set of experiments, we were interested in the effect of various microRNAs on the tumor suppressor gene LATS2. Fig. 11.13 shows some of the data from the optimizing experiments for LATS2 and its putative miRNA regulator using the protocol outlined previously. As is evident, no signal was generated for LATS2 in the normal lung if no pretreatment (panel A) or protease digestion (panel B) was used. A strong signal was evident with antigen retrieval with no detectable background (panel C). Note that the signal stays sharply localized to the mononuclear cells. If antigen retrieval and protease digestion were used, the same cells in the serial section showed a strong signal, but background was now evident. The background is evident as the relatively weak red staining over the stroma (arrows). Thus, we can conclude that, in the coexpression experiments, LATS2 detection will be optimized by antigen retrieval. Now look at the data for the miRNA after in situ hybridization optimization. A strong signal is noted with protease digestion (panel E).
background with miRNA in situ hybridization detection; the intense signal localizes to a specific cell type (cancer cells, large arrow) and is not evident in an adjacent different cell type (normal lung, small arrow). Compare this to panel F, where the miRNA was detected after RNA retrieval. The signal is evident but is less distinct, being present in the cancer cells and surrounding stromal cells. If RNA retrieval was followed by protease digestion, then background became very high (data not shown). Thus, the optimizing experiments indicated that the microRNA would be best detected with protease digestion.

We then analyzed serial sections for LATS2 (antigen retrieval) and the miRNA (protease digestion). The slide tested for LATS2 first by antigen retrieval was then tested for the miRNA by in situ hybridization with no additional pretreatment. The slide tested for the microRNA by in situ hybridization after protease digestion was then tested for LATS2 using antigen retrieval. In each case, the miRNA signal was NBT/BCIP (blue), and the LATS2 signal was red due to Fast Red.

As evident in Fig. 11.14, rare cancer cells showed LATS2 signal (panel A). Note the strong signal for LATS2 in the same tissue for a benign bronchiole (insert), which serves as an internal positive control. The LATS2 signal is seen in both the cytoplasm and the nucleus. In comparison, many more cancer cells were positive for the miRNA (panel B) where, again, a nuclear and cytoplasmic signal were each evident. When the microRNA in situ hybridization was done first, followed by the LATS2 detection (panel C), each signal could easily be recognized by routine microscopy. This is a situation in which the computer-based system is invaluable; there are nests of cancer cells where both the microRNA and its putative target are present. Note that the coexpression analysis (panel D) did not demonstrate any coexpression, which, thus, is consistent with the theory that the microRNA may be regulating the LATS2 protein. Look at the data if the immunohistochemistry for LATS2 was done first, followed by the microRNA (panel E). The signal for the LATS2 is still evident, but the microRNA signal is no longer evident, as the RNA retrieval was simply not the correct pretreatment to optimize the signal, as we saw in Fig. 11.13. Thus, the coexpression data are not useful because only the LATS2 signal is present (panel F).

Remember that, with a good knowledge of the cytopathology and histopathology and the subcellular localization of the targets, you can detect even four (Fig. 11.15, panels A−C) or five (panels D−F) antigens in a given tissue using just one chromogen. Of course, in this case, each target had the same optimization profile. I never cease to be amazed as to how “clean” the in situ hybridization and immunohistochemistry is at the biochemical

![FIGURE 11.12 Coexpression of EBV RNA and protein in lymphoma cells. Panel A shows a diffuse large B-cell lymphoma that was analyzed for both EBER-1 and -2 by in situ hybridization (blue signal; separated in panel C), and the EBV protein bzlf-1 by immunohistochemistry (red signal, separated in panel B). Each target is nuclear-based, and thus, you must do coexpression analyses to determine which cancer cells are expressing both targets. The Nuance system (panel D) clearly shows that the cancer cells are making both EBER-1 and -2, and bzlf-1, which is indicative of lytic infection.](image-url)
FIGURE 11.13 Coexpression of LATS2 and a microRNA: importance of optimizing pretreatment conditions with serials sections: Part A. Panel A shows that no signal was generated for LATS2 in the normal lung if no pretreatment (panel A) or protease digestion (panel B) was used. A strong signal was evident with antigen retrieval with no detectable background (panel C). Note that the signal stays sharply localized to the mononuclear cells. If antigen retrieval and protease digestion was used, then the same cells in the serial section showed a strong signal, but background was now evident. The background is evident as the relatively weak red staining over the stroma (arrows). Compare this to the optimizing data for the miRNA that may regulate LATS2. A strong signal is noted with protease digestion (panel E). Note the typical features of a strong signal and minimal background with miRNA in situ hybridization detection; the intense signal localizes to a specific cell type (cancer cells, large arrow) and is not evident in an adjacent different cell type (normal lung, small arrow). Compare this to panel F where the miRNA was detected after RNA retrieval. The signal is much less distinct. Thus, the coexpression experiment would be best done with the miRNA first (protease digestion) followed by LATS2 after antigen retrieval; remember to use a lower concentration of the primary antibody as discussed in Chapter 5, The Basics of Immunohistochemistry, because this will reduce the background seen in panel D.
level, in that you can easily run many separate reactions simultaneously.

Fig. 11.16 shows the value of performing coexpression analyses on serial sections. The tissue is a lung cancer. Serial section 1 was tested for miR-34a; note that most of the cancer cells are negative (panel A). Panel B is the next serial section tested for PDGFR-β, and panel C is the subsequent serial section tested for PDGFR-α. It is clear from these data that miR-34a is not being expressed in the cancer cells, but that most are expressing both forms of PDGFR. This is confirmed in panel D, where there is no evident coexpression when the Nuance system analyzed miR-34a and PDGFR-β in the same tissue section.

The second edition of this textbook was written during the COVID-19 pandemic. Thus, let’s show the value of coexpression analyses in understanding the mechanism of SARS-CoV-2 induced lung damage. Note in Figure 11.17 the regular light microscopy image when in situ hybridization for SARS-CoV-2 (brown) is done on the same tissue as the alveolar macrophage marker CD11b (red). Using the Nuance software, the viral RNA signal is now
green and the CD11b signal is red with co-expression marked by yellow. One can see with the co-expression experiments that some of the viral RNA is in macrophages whereas the rest is in the endothelial cells of the alveolar septa. Indeed, the alveolar macrophage is the primary target of the virus in the lung as evidenced by the in situ based data.

Coexpression of SARS-CoV-2 RNA with the alveolar marker CD11b. The light microscopy panel (right) shows viral RNA as brown and CD11b as red. The Nuance derived image shows the viral RNA as green and macrophage marker as red (left). Note that the virus infects both the alveolar macrophage and the adjacent septal capillary endothelial cells, which in turn can be demonstrated with CD31 co-expression.

Indeed, it was the in situ based methods that, as demonstrated in large part by Dr. Cynthia Magro at Cornell Medical Center, that the pathophysiology of severe COVID-19 disease has three key points: 1) massive lung infection that causes microthrombi (microangiopathy) in
FIGURE 11.16 The value of serial sections in coexpression analyses. Serial sections for this lung cancer were tested for miR-34a (panel A), PDGFR-β (panel B), and PDGFR-α (panel C). It is clear that the cancer cells are expressing abundant PDGFR-α and -β but not the miR. Indeed, extensive additional analyses including the luciferase assay and mutational analyses (by Dr. M Garofalo) showed that the miRNA was regulating these two forms of PDGFR. Panel D confirms the lack of coexpression of miR-34a and PDGFR-β (panel D) (the miRNA is fluorescent blue, the PDGFR-β is fluorescent green, and the coexpression would be seen as fluorescent yellow).

FIGURE 11.17 The second edition of this textbook was written during the COVID-19 pandemic. Thus, let’s show the value of coexpression analyses in understanding the mechanism of SARS-CoV-2 induced lung damage. Note in Figure 11.17 the regular light microscopy image when in situ hybridization for SARS-CoV-2 (brown) is done on the same tissue as the alveolar macrophage marker CD11b (red). Using the Nuance software, the viral RNA signal is now green and the CD11b signal is red with co-expression marked by yellow. One can see with the co-expression experiments that some of the viral RNA is in macrophages whereas the rest is in the endothelial cells of the alveolar septa. Indeed, the alveolar macrophage is the primary target of the virus in the lung as evidenced by the in situ based data.
the septal capillaries that destroys both the virus and the infected alveoli; 2) release of circulating viral capsid proteins that hone onto endothelial cells; 3) that by far the most common site of ACE2+ endothelial cells is the skin and adjacent subcutaneous fat. Viral capsid protein attaching to these endothelial cells induce the massive cytokine storm and increased coagulable state typical of the fatal disease. The latter observation easily explains why obesity is such a strong risk factor for fatal COVID-19.