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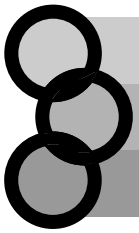

ORAL SURGERY
ORAL MEDICINE
ORAL PATHOLOGY

*ORAL AND MAXILLOFACIAL PATHOLOGY* *Editor: Alan R. Gould*

# Advanced diagnostic methods in oral and maxillofacial pathology. Part I: Molecular methods

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The practice of pathology is currently undergoing significant change, in large part due to advances in the analysis of DNA, RNA, and proteins in tissues. These advances have permitted improved biologic insights into many developmental, inflammatory, metabolic, infectious, and neoplastic diseases. Moreover, molecular analysis has also led to improvements in accuracy of disease diagnosis and classification. It is likely that, in the future, these methods will increasingly enter into the day-to-day diagnosis and management of patients. The pathologist will continue to play a fundamental role in diagnosis and will likely be in a pivotal position to guide the implementation and interpretation of these tests as they move from the research laboratory into diagnostic pathology. The purpose of this 2-part series is to provide an overview of the principles and applications of current molecular biologic and immunologic tests. Part I will discuss the biologic fundamentals of DNA, RNA, and proteins and the methods that are currently available or likely to become available to the pathologist in the next several years for their isolation and analysis in tissue biopsies. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001;92:650-69)**

The analysis of DNA, RNA, and proteins, obtained from diagnostic specimens, is currently revolutionizing the practice of surgical pathology and heralds a new era of diagnostic and prognostic tests that will greatly influence our day-to-day clinical decision making. The diagnosis of cancer and many other diseases is fundamentally based on the microscopic study of cells and tissues. This diagnostic method remains the standard by which all other diagnostic tests are measured. Nevertheless, the era of the pathologist relying entirely on the examination of tissue sections stained by histochemical methods is gradually being replaced by a time when advanced immunologic and molecular techniques (ie, analysis of DNA, RNA, or protein structure or function) augment the process by which complicated infectious, inflammatory, metabolic, and neoplastic diseases are diagnosed and classified. Many of these molecular advances had their start in basic science research laboratories; after validation, technological improvements, and automation, they have made their way into applied molecular pathology research and increasingly into the day-to-day practice of pathology. Today, the extent to which immunopathologic and molecular pathologic techniques are used varies greatly, but it is conceivable that in the next decade many of today's most technically advanced methods of molecular analysis will become standard practice. Part I of this series will provide an overview of many of the most important tools emerging in diagnostic pathology, including the polymerase chain reaction (PCR), in situ hybridization (ISH), complementary DNA (cDNA) microarrays, flow cytometry, and laser microdissection.

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## MOLECULAR METHODS IN DIAGNOSTIC PATHOLOGY

### Principles of molecular pathology

The basic building blocks for all eukaryotic life reside in the genes of cells. Genes, composed of deoxyribonucleic acid (DNA), encode for proteins that are the principal effector molecules for all biologic processes. Although DNA was first isolated in 1869 by Johann Friedrich Miescher, its structure was not known until

1953, when Watson and Crick published evidence of the double helix.[1] DNA is a highly coiled, double-stranded molecule located within the nucleus of eukaryotic cells. It is composed of an ordered sequence of nucleotides, each consisting of the sugar deoxyribose, a phosphate group, and 1 of 4 bases, as follows: adenine (A), thymine (T), guanine (G), or cytosine (C). Each single strand of DNA binds, in a complementary fashion, with its opposing strand by adenine-thymine (A-T) and guanine-cytosine (G-C) pairing. Nucleotide pairing is highly precise, thus information represented by the genetic code is contained in each strand.

Protein synthesis proceeds through a series of defined steps, the first being the transcription of DNA into ribonucleic acid (RNA). *Transcription* is the term describing the base-pairing process that copies DNA into complementary RNA. Unlike DNA, RNA is single-stranded, consisting of the sugar ribose, a phosphate group, and the nucleotide bases adenine, guanine, and cytosine, with, in place of thymine, uracil (U). RNA is formed in the nucleus by complementary binding of RNA nucleotides to a single strand of DNA, which acts as a nucleotide sequence template. With transport of the RNA molecule to the cytoplasm and some modification, the molecule is termed *messenger RNA* (*mRNA*). A protein is produced on the mRNA template by the assembly of a string of amino acids, a process termed *translation*. The sequence of nucleotide base triplets (codon) in the mRNA dictates the order of specific amino acids, linked together to form a protein. Proteins are the effector molecules of all cells, and their amino acid sequence and tertiary structures, to a large degree, are determined by the nucleotide sequences contained in the nuclear DNA of the cell.

## PCR-based diagnostics

*Principles.* PCR has emerged as one of the most powerful tools for the amplification of genes and their RNA transcripts. Although the technique is relatively new, having been first described in 1985,[2] it was alluded to conceptually at least 30 years ago.[3] The identification of a heat-stable DNA polymerase and the development of machines to automate the repetitive heating and cooling cycles needed for the technique have greatly improved the methodology. Today, PCR is the single technique that is used almost universally to permit the study of DNA and RNA obtained from a variety of tissue sources.

PCR typically begins with the isolation of DNA (genomic DNA) from a fresh tissue specimen or from tissue in a paraffin block. If RNA is the object of analysis, it must first be converted to cDNA through reverse transcription. Heating DNA separates the complementary double strands into single-stranded forms intended to act as a template dictating the nucleotide sequence in vitro. Two short oligonucleotide primers are designed to anneal (bind) to the template and flank a region of interest (Fig 1). A thermostable DNA polymerase, isolated from the *Thermus aquaticus* organism, known as Taq polymerase, catalyzes the sequential addition of the 4 nucleotides (deoxynucleotide triphosphates [dNTPs]) to the primers.[4] Cooling the solution permits the primers to bind to the template DNA, and then the Taq polymerase catalyzes the addition of dNTPs to the template between the primers. Salts and buffers permit the Taq polymerase to catalyze the reaction. This process is then repeated, and with each cycle there is an exponential increase in the quantity of DNA such that after $n$ cycles, the amount increases by $2^n$. A typical PCR application involves 30 to 40 cycles; therefore, after 35 cycles, $3.44 \times 10^{10}$ ($2^{35}$) copies of the template DNA have been made.

Although PCR is a powerful method to increase the amount of a gene of interest in vitro, it has a number of important limitations. Difficulties can be encountered when studying small quantities of DNA, because the ingredients necessary for PCR (ie, oligonucleotide primers, dNTPs, Taq polymerase) may be exhausted before sufficient target is produced. The specificity of the reaction may be limited and depends on many complex, interrelated factors, including oligonucleotide primer size, annealing temperature, and the buffer salt concentration. Long DNA fragments (>300 base pairs) are difficult to amplify when the starting material is degraded such as may be produced by formalin fixation. A major limitation of PCR is the susceptibility of the process to contamination, particularly in experiments intended to detect rare DNA sequences and account for unusual and unexpected results.[5] With "clean" laboratory techniques such as those using disposable laboratory-wear and with appropriate sample controls, this problem can be overcome.[6] Finally, on occasion, the Taq polymerase may produce nucleotide addition errors, because the enzyme has no "proofreading" properties. Moreover, nucleotide sequence errors are more likely when the template DNA is fragmented or degraded, such as that obtained from formalin-fixed, paraffin-embedded tissue sections. This is of particular concern when the target fragment is destined for determination of the nucleotide sequence.[7]

Modifications have been developed to increase the specificity of the PCR. For example, the "hot-start" technique involves the addition of a key ingredient, such as the Taq polymerase, after the first denaturation step, preventing nonspecific binding and extension below the optimal annealing temperature. Nested PCR

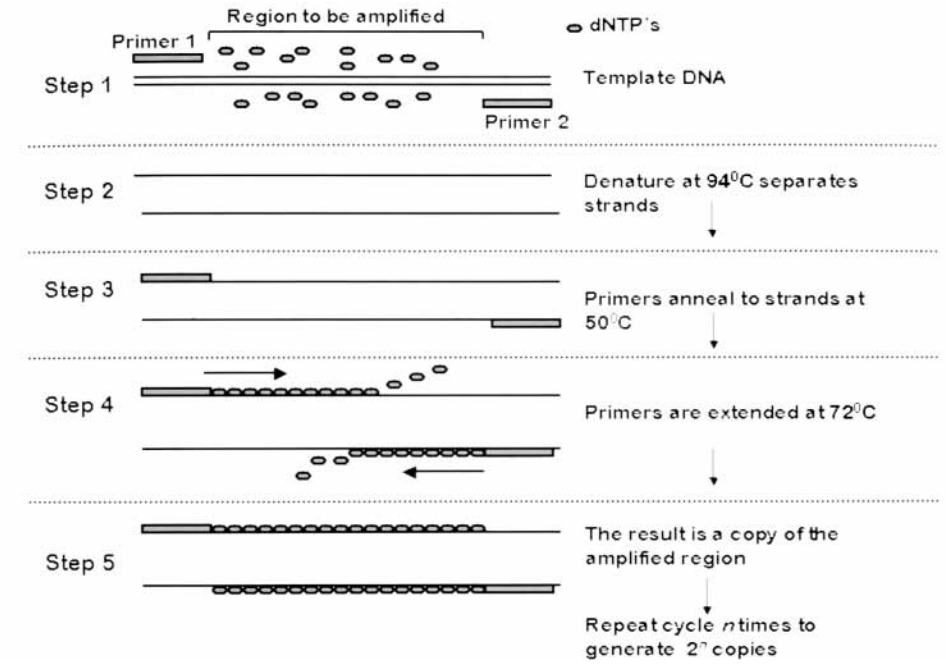


Fig 1. The basic steps for a single cycle of PCR. *Step 1:* The reaction mixture contains genomic (template) DNA with a target region to be studied, nucleotides (dNTPs), 2 primers, and buffer. *Step 2:* The mixture is heated to 94°C to separate the double strands. *Step 3:* When the temperature is then lowered to 50°C, the primers bind to the DNA template surrounding the region to be studied. *Step 4:* Raising the temperature to 72°C permits the nucleotides to be added along the template DNA. *Step 5:* The result of this one cycle is 2 copies of the region of DNA of interest. The steps are then repeated $n$ times to generate $2^n$ copies of the DNA region of interest.

increases specificity; the reaction is performed with one set of primers followed by reamplification using a second set of primers complementary to sequences within the first product.[8] Nested PCR techniques are also useful for detecting rare sequences in a larger pool of DNA, such as for determining evidence of cellular derivation from a single progenitor cell (monoclonality), a characteristic of specific neoplastic diseases such as lymphoma.[9]

*Materials for analysis.* Many different types of clinical samples have been used for PCR analysis, including blood, saliva, sputum, semen, and single hairs.[10-12] Whereas the traditional methods for genetic analysis such as Southern blotting (a technique to analyze DNA whereby the DNA is size-fractionated by gel electrophoresis, transferred to a nylon membrane, and then hybridized with a labeled probe; see "Hybridization methods") and Northern blotting (similar to Southern blotting but for analysis of RNA) require relatively large amounts of high-quality DNA or RNA, PCR can be used to amplify relatively degraded DNA from a variety of sources, including DNA extracted from paraffin-embedded tissue sections.[13,14]

The tissue fixative 4% neutral-buffered formalin causes cross-linking and cuts in the DNA; the latter alteration is termed *nicking*. Although proteolytic digestion of tissues produces large quantities of DNA, most is fragmented—limiting the size of the target area of the gene that can be subjected to PCR.[7] Precipitating fixatives such as ethanol and acetone do not cross-link or shear the DNA and, therefore, produce more consistent PCR results.[15] Longer fixation times (>24 hours) may also adversely affect the quality of the DNA extracted from routinely processed tissues and thereby reduce PCR efficiency.

The counterstain used for tissue visualization before DNA extraction can also affect the quality of the reaction. For example, a recent study found that PCR efficiency was highest when methyl green and neutral fast red counterstains were used, with PCR efficiency comparable to that obtained with unstained sections.[16] By contrast, hematoxylin often produced significantly poorer PCR results than other stains. The basis for hematoxylin's adverse effect on PCR was uncertain, but it is possibly related to stain binding to DNA phosphate groups and to increased resistance to protease digestion.

*Applications*

MICROBIOLOGY. The use of PCR has revolutionized the

**Table I.** Comparison of in vitro methods used to study nucleic acids*

| *Methods* | *Template* | *Detection* | *Advantages* | *Disadvantages* |
|---|---|---|---|---|
| PCR | 10-25 ng of genomic DNA but as little as picogram quantities may be used | • Analytical gel and fluorescent staining<br>• Radioactive | • Extremely sensitive<br>• Rapid, specific<br>• Flexible and adaptable | • Technically demanding<br>• Often too sensitive, false-positive<br>• Contamination |
| Reverse transcription | <1 ng total RNA<br><1 × $10^4$ cells | • Analytical gel and fluorescent staining<br>• Radioactive | • Extremely sensitive<br>• Rapid, specific<br>• Flexible and adaptable | • In contrast to Northern blotting, no mRNA size information<br>• Technically demanding<br>• Often too sensitive, false-positive<br>• Contamination |
| Southern blot | 10-20 μg of genomic DNA | • Radioactive<br>• Colorimetric<br>• Chemiluminescence | • DNA size information<br>• Quantification of gene copy number possible | • Time-consuming<br>• Requires transfer to a membrane<br>• Requires hybridization<br>• Much high-quality DNA needed |
| Northern blot | 5 μg of poly (A)+ mRNA<br>10-20 mg of total RNA | • Radioactive<br>• Colorimetric<br>• Chemiluminescence | • mRNA size information<br>• Accurate quantification of mRNA expression | • Time-consuming<br>• Requires transfer to a membrane<br>• Requires hybridization<br>• Much high-quality RNA needed |

*Adapted with permission from Tsongalis and Coleman.[92]

diagnosis and study of infectious diseases and malignancies associated with microorganisms. In the past, the diagnosis of many infectious diseases was difficult, often hampered by the supply of material for microbiologic culture, protein analysis, or direct microscopy. Moreover, culture and identification techniques typically require significant time and are labor-intensive.

PCR overcomes many of these problems and in some cases has replaced traditional pathogen identification methods. DNA or RNA of an infectious organism can be detected in test material even when organism number is low or is slowly growing or when the infectious agent is in material not suitable for culture.[17] Examination of archival material has permitted retrospective studies establishing the role of infectious organisms in the etiology and pathogenesis of many neoplasms, including human papillomavirus in cervical carcinoma, Epstein-Barr virus in posttransplant malignancies, and human herpesvirus 8 in Kaposi's sarcoma.[18,19] The list of infectious agents that can be detected by PCR is extensive, and the technique has been used to detect organisms in blood, saliva, sputum, semen, and feces, as well as in fixed tissues.[20-22]

HUMAN GENETICS. PCR plays an important role in the identification of chromosomal disorders and hereditary diseases, including cystic fibrosis, Gaucher's disease, alpha-1-antitrypsin deficiency, hemophilia, and sickle cell anemia. PCR can also be used to analyze fetal DNA for aneuploidy (the presence of extra chromosomes or the absence of chromosomes), trisomy 21, Turner's syndrome, Klinefelter's syndrome, and for sex determination.[23-25]

FORENSIC PATHOLOGY. Forensic pathology has used PCR in a range of situations, including for the identification of mutilated or decomposed human tissues, for sex determination, and for disputed paternity cases. "DNA fingerprinting" is based on the identification of variable tandem repeats, short, repeating DNA nucleotide sequences that litter the human genome. This process has proved to be an important tool in the identification of criminals.[26,27]

TUMOR BIOLOGY. PCR has revolutionized the study of cancer and provided greater insights into the pathobiology of neoplasia. PCR has been used to detect mutations in cancer-associated oncogenes (eg, K-*ras,* N-*ras*),[27] tumor suppressor genes (eg, p53, p16),[28-30] monoclonality in B- and T-cell lymphomas (Fig 2),[14] chromosomal translocations such as the Philadelphia chromosome t(14;18) in chronic myelogenous leukemia, and minimal residual neoplastic disease.[31] The most profound impact of PCR has been on the study of genetic changes in formalin-fixed tissues. For example, the tumor suppressor gene p53 is frequently inactivated by single nucleotide substitutions, a form of gene mutation. Identification of the substitutions entails using PCR to increase and isolate coding regions

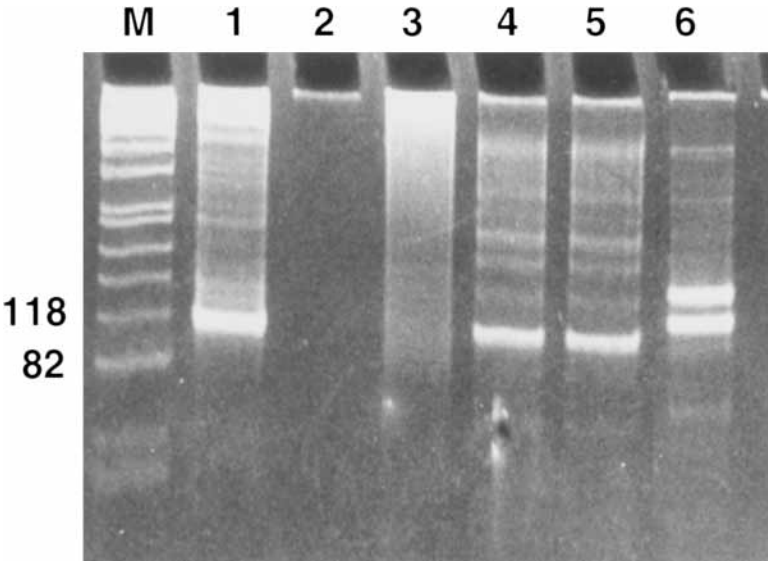


Fig 2. The application of PCR to identify heavy-chain gene monoclonality in a lymphocyte population. Here, the immunoglobulin heavy-chain gene was subjected to PCR in a series of labial salivary gland biopsies from patients with Sjögren's syndrome. Normally, if the lymphocyte population is polyclonal, a smear will be seen. If there is a monoclonal population, single or double bands will be seen in gel electrophoresis. *Lane M,* Molecular size marker indicating 82 and 118 base pairs. *Lane 1,* DNA from a lymphoma showing a single band. *Lane 2,* Negative control. *Lane 3,* A polyclonal (non-neoplastic) population of lymphocytes. *Lanes 4, 5, and 6,* Monoclonal populations of lymphocytes indicate that the lesion is likely neoplastic.

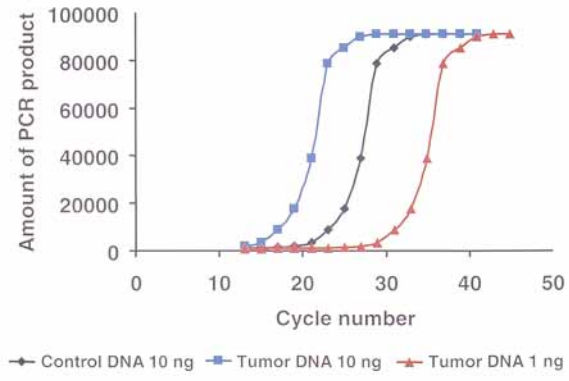


Fig 3. Graph depicting the kinetics of PCR. Early in the reaction there is little detectable product; however, as the cycle numbers increase, PCR product accumulates in an exponential manner. In this example, for 10 ng of tumor DNA, this phase occurs between cycles 20 and 25. As primer, Taq polymerase, and buffer are exhausted, the amount of PCR product no longer accumulates in an exponential manner and reaches a saturation phase. The amplification curves are also influenced by the amount of DNA present in the mixture. Large quantities of starting DNA result in a more rapid accumulation of PCR product than smaller quantities of starting DNA (10 ng vs 1 ng of tumor DNA).

followed by sequencing of the product.[32] Finally, PCR has been used as a screening technique for detection of malignant cells in human secretions. Because PCR is ideally suited to the study of low numbers of unique DNA fragments, it has been applied to the detection of malignant cells in urine, sputum, and saliva.[33]

*Quantitative PCR.* Quantification of DNA by PCR has tremendous potential because it can permit the determination of gene amplification (a mutational event whereby a gene is found in greater numbers than the normal number of copies) of a myriad of genes (Table I). Potentially, quantitative PCR offers a number of advantages over traditional gene quantification methods such as Southern blot analysis. For example, the exponential increase in DNA during PCR cycling permits the use of relatively small amounts of genetic material that may be fragmented or degraded. Moreover, PCR methods can be automated to permit analysis of large sample numbers relatively easily, providing a measure of flexibility not permitted by the conventional laborious and time-consuming methods.

In practice, however, a number of technical challenges had to be overcome to develop reliable, quantitative PCR. One of the principal challenges is the nature of PCR product accumulation. During the reac-

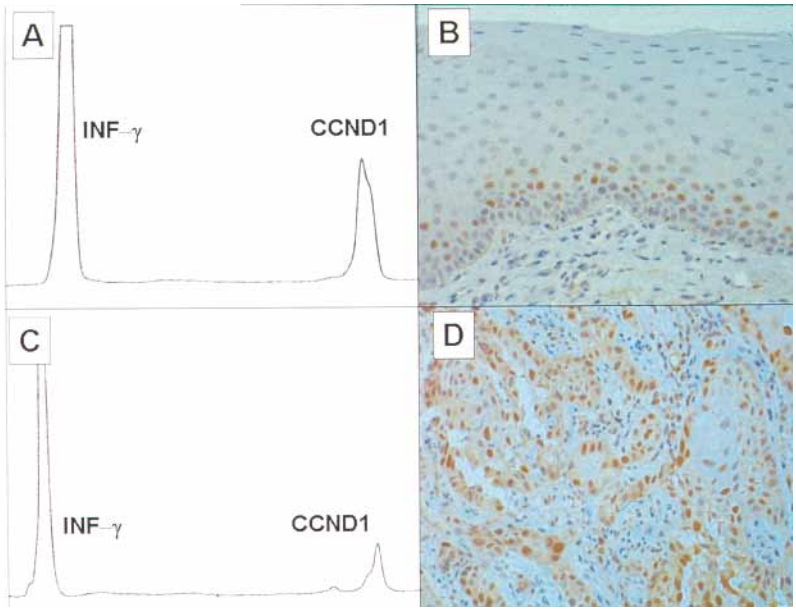


Fig 4. Quantitative duplex PCR for the *CCND1* (cyclin D1) gene amplification in normal mucosa and oral SCC. For each sample (*panels A & C*) the *CCND1* (cyclin D1) gene was subjected to PCR in the same reaction with the *interferon-γ (INF-γ)* control gene. The area under each peak represents the amount of PCR product present at the mid-point in the reaction. **A,** Normal mucosa: The ratio of *CCND1* to *INF-γ* is 0.8, indicating no amplification of the *CCNDI* gene. **B,** Normal mucosa: Immunohistochemistry shows no overexpression of cyclin D1 protein in this tissue (original magnification ×400). **C,** Oral SCC: The ratio of *CCNDI* to *INF-γ* is 4.3, indicating gene amplification. **D,** Oral SCC: Immunohistochemistry of this tumor shows overexpression of cyclin D1 protein (original magnification ×200).

tion there are 2 defined phases whereby product increases. At low cycle numbers, PCR product accumulates exponentially (exponential phase), but at higher cycle numbers, as template DNA, dNTPs and primer are consumed, the rate of product formation progressively decreases until none is formed (saturation phase; Fig 3). The detectable exponential phase may be relatively short, lasting only a few cycles. Moreover, making a determination of the beginning and the end of this phase may be difficult because it is affected by many factors, including amount and quality of template DNA and the kinetics of the reaction. To perform quantitative PCR, measurements of the amount of product must be made during the exponential phase of the reaction, because measurements during the saturated phase will provide inaccurate results.

To circumvent these problems, a number of methods have been devised. One strategy is to use a duplex PCR technique whereby the gene of interest is subjected to PCR with a reference (nonamplified, control) gene together in the same PCR (Fig 4). In this way the relative amount of the target gene can be compared with the amount of the reference gene at a fixed point in the reaction. The method has proved valuable for the retrospective study of gene amplification in human tumors, including c-*erbB-2 (HER2/neu)* in salivary gland[34] and breast tumors,[35] *int-2* in thyroid cancers,[36] and *CCND1* (cyclin D1) in breast carcinomas,[37] oral cancers,[38] and epithelial dysplasias.[39]

At present, the method of choice for quantitative PCR is continuous monitoring of the amount of product at the end of each cycle. There are a number of ways to accomplish this, including the TaqMan PCR 5´ nuclease assay. This was first described by Holland et al,[40] who used the 5´-3´ endonuclease activity of Taq DNA polymerase to detect target sequences during PCR. Included in the PCR mixture is a short oligonucleotide probe designed to hybridize within the target sequence but not to be extended in the 3´ direction (by convention, the downstream portion of a DNA strand). When the probe is hybridized to the target, no signal is produced because of the presence of a quencher molecule that suppresses the fluorescence of a reporter molecule on the probe. During PCR, the probe hybridizes to the target DNA, and Taq polymerase cleaves the probe into shorter fragments, thereby releasing the quencher from the reporter molecule. The

amount of fluorescence generated is, therefore, directly proportional to the amount of PCR product generated. By measuring the amount of PCR product produced at the end of each single cycle, PCR growth curves can be plotted and measurements taken from the exponentially expanding region of the reaction.

*Reverse transcriptase PCR.* Another important application of PCR has been the detection and quantification of mRNA in cells. Because mRNA is short-lived and unstable, the determination of its relative abundance is often difficult in tissue sections by using conventional methods of RNA analysis such as Northern blotting (see "Hybridization methods"). Moreover, the proportion of mRNA in the total amount of RNA in a cell (composed of ribosomal RNA, transfer RNA, and mRNA) may be as little as 2%, making the identification of specific mRNA species challenging. The analysis of mRNA is important because it provides direct evidence of cell transcription and, therefore, is a measure of cellular function.

The basis of reverse transcriptase (RT)–PCR is the conversion of RNA to DNA. cDNA is that which is transcribed from an RNA template by the enzyme RT. This enzyme functions in an analogous—but reverse—manner to the way RNA is made from a DNA template by RNA polymerases. Thus, an RNA nucleotide sequence of GGUUA is directly converted by RT to CCAAG in cDNA. This cDNA can then be used as a template for PCR or quantitative PCR. If the starting material were mRNA, then the resultant cDNA would contain only exons (the parts of the gene that are found in the mRNA molecule that will be used to code for the protein) and not introns (the part of the gene that is not found in the transcribed mRNA) because these are spliced out when mRNA is made.[41]

An important application of RT-PCR has been in the detection and quantification of the transcripts of tumor-associated translocations. Many neoplasms—particularly hematopoietic malignancies—contain specific chromosomal translocations. For example, the Philadelphia chromosome is a genetic alteration that is most commonly identified in chronic myeloid leukemias and a subset of acute lymphoblastic leukemias. This is the result of a reciprocal translocation between chromosome 9 and chromosome 22, causing the relocation of the proto-oncogene c-*abl* (from chromosome 9) adjacent to the c-*bcr* gene on chromosome 22 [t(9:22)]. This produces a hybrid c-*abl*-*bcr* transcript (mRNA) that encodes for a chimeric protein with tyrosine kinase activity. RT-PCR can be used to detect the fusion transcript when PCR primers are designed to flank the translocation. Quantitative PCR methods can also be applied to the cDNA of the fusion transcript to provide a measure of the amount of mRNA present in a neoplastic cell. As will be discussed later, this strategy can also be used for the posttreatment detection of minimum residual disease in patients with leukemia.[42] Other tumor-defining translocations can be detected by RT-PCR, including t(15;17) in acute promyelocytic leukemia, t(8;14) in Burkitt's lymphoma, t(2;5) in anaplastic large-cell lymphoma, t(11;22) in Ewing's sarcoma and primitive neuroectodermal tumor, t(2;13) in alveolar rhabdomyosarcoma, and t(X;18) in synovial sarcoma.[43-45]

*DNA sequencing methods.* The most precise description of a gene is delineation of its nucleotide sequence. Because a mutational event is required for the inactivation of most tumor suppressor genes, the goal of many tumor studies is the determination of its DNA sequence. Sequencing methods have been used for several decades, but improvements in technology have reduced testing time to several hours and have permitted characterization of genes composed of up to hundreds of thousands of nucleotides.

Two methods have been developed to sequence DNA. In 1977, Maxam and Gilbert[46] described a method involving chemical degradation of radioisotopically labeled DNA at susceptible sites that are then analyzed by gel electrophoresis (the movement of charged molecules toward an electrode of the opposite charge; used to separate DNA, RNA, and protein by size). Today, most manual and automated sequencing methods are based on the method described by Sanger et al,[47] relying on the generation of complementary single-stranded DNA using DNA polymerase. Here, 4 mixtures are prepared, each containing DNA polymerase and all the deoxynucleotide triphosphates (dATP, dCTP, dTTP, and dGTP; Fig 5). One of the nucleotide precursors is labeled with a radioactive or fluorescent marker in each mixture. In addition, each of the 4 reaction mixtures also contains a limiting amount of one dideoxynucleotide (ddATP, ddCTP, ddTTP, or ddGTP), which, when incorporated into the DNA, causes premature chain termination. Therefore, in the reaction tube containing the ddGTP, nucleotides will be added to the complementary strand until a ddGTP is added. When this occurs, chain elongation ceases because ddGTP (or ddCTP, ddATP, or ddTTP, depending on the reaction tube) lacks the 3´-hydroxyl group required for subsequent nucleotide addition. Thus, the reaction-tube mixture containing the ddGTP will generate multiple strands of DNA of various lengths, all terminating at positions where ddGTP has been incorporated. Similarly, the other 3 tubes will all contain DNA fragments of various lengths terminating at positions where the respective dideoxynucleotide has been added. The contents of each of the 4 tubes are individually separated by means of gel electrophoresis, with each band produced corresponding to the size of

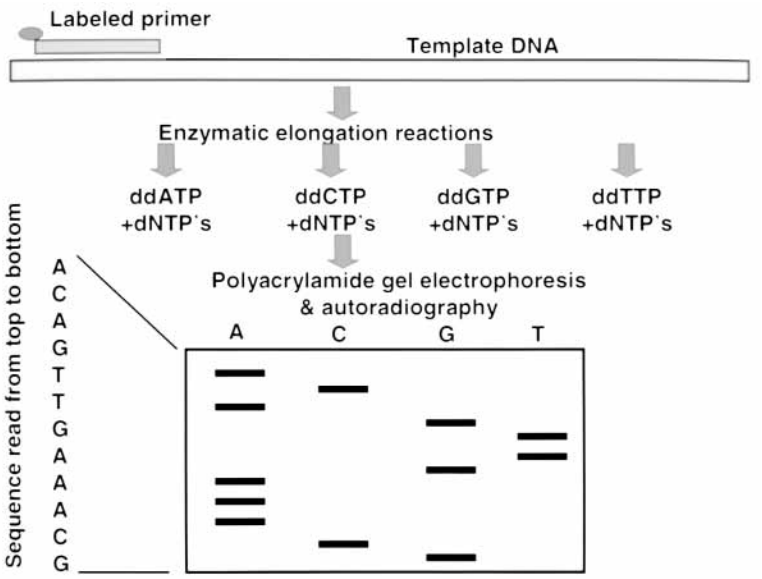


Fig 5. The sequencing of DNA requires a template DNA and a labeled primer at one end of the region to be sequenced. Four mixtures are prepared, each containing the DNA polymerase enzyme and the nucleotides (dATP, dCTP, dTTP, and dGTP). Each of the 4 reactions also contains a limiting amount of one dideoxynucleotide (ddATP, ddCTP, ddTTP, or ddGTP). When a dideoxynucleotide is added, chain elongation ceases. Therefore, in the reaction tube that contains the ddGTP, nucleotides including dGTP will be added. The contents from each of the 4 tubes are then separated in 4 lanes by gel electrophoresis, and bands are produced corresponding to the size of the terminated fragments. The sequence is then read from top to bottom so that, in this gel, the sequence is ACAGTTGAAACG. Adapted from Tsongalis and Coleman,[92] by courtesy of Marcel Dekker Inc.

the terminated DNA fragments. Because smaller fragments travel faster in the gel, those fragments that were terminated early by addition of the dideoxynucleotide will migrate farthest in the gel.

In the past, DNA sequencing was performed by using laborious and time-consuming manual techniques. Today, this process is often automated by using fluorescence-labeled nucleotides read by a laser during passage through an electrophoresis sequencing gel (Fig 6). The fastest methods available involve sequencing analysis by capillary electrophoresis whereby parallel, tiny fiberoptic glass tubes contain a special polyacrylamide sieving medium for separation of fluorescence-labeled DNA fragments generated by the sequencing reaction.[48]

## Hybridization methods

*Hybridization* refers to the pairing of complementary RNA or DNA strands to produce a double-stranded nucleic acid. The nucleotide base-pair relationship is so specific that strands cannot anneal unless the respective nucleotide strand sequences are complementary. All hybridization methods use a radio-labeled or fluorescence-labeled DNA or RNA probe that binds to the target DNA or RNA of interest, permitting visualization. The target nucleic acids can either be immobilized in a membrane ("blotting") or examined in tissue sections (in situ).

*Southern blotting.* A widely used method for analyzing the structure of DNA is that described by Southern in 1975 (Table I).[49] This involves the transfer or blotting of DNA fragments onto a membrane (Fig 7). DNA is first enzymatically cleaved into smaller pieces by restriction endonucleases (enzymes that are able to cut DNA at specific recognition sites), then size-separated by agarose gel electrophoresis. Smaller fragments travel farther in the gel, moving away from the negatively charged cathode, whereas larger pieces migrate a shorter distance. Thus, electrophoresis serves to separate fragments according to size, a process termed *fractionation*. After fragment separation, the DNA is transferred from the gel to a nylon or nitrocellulose membrane through the capillary action of a buffer as it is absorbed by blotting paper. Then, the DNA is bound to the membrane by baking the membrane in a vacuum oven or by ultraviolet light cross-linking. Finally, specific DNA fragments can be identified by hybridizing the membrane with labeled

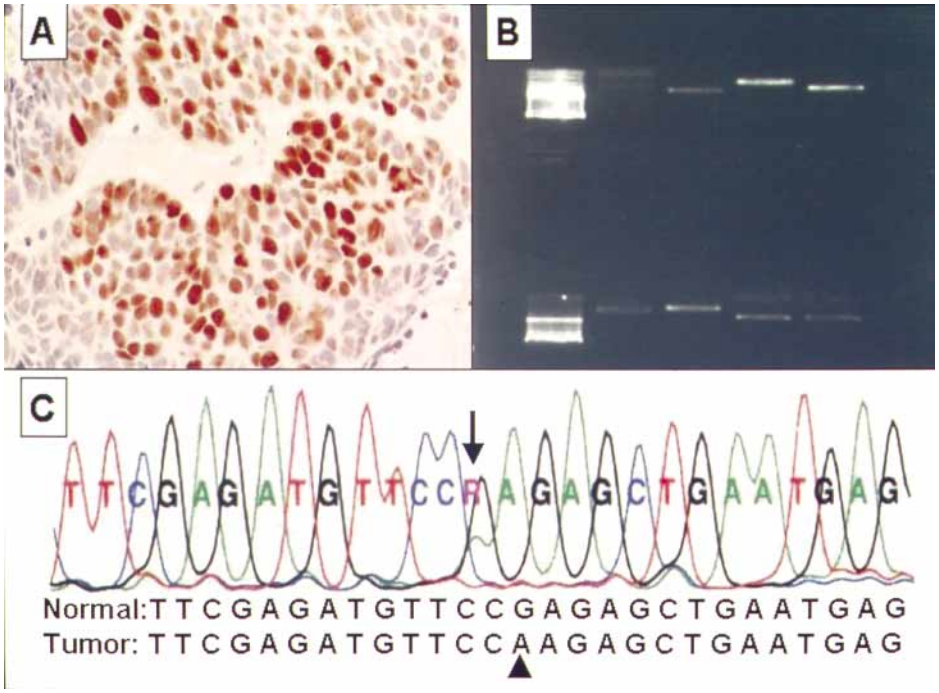


Fig 6. Identification of p53 alterations in an oral cancer. **A,** Ordinarily, p53 is undetectable in tissue sections because the half-life of the protein is too short to detect *(nuclear staining: blue).* However, mutant or stabilized wild-type p53 can be identified in the nuclei of oral carcinoma cells by immunohistochemistry. *Brown nuclear staining* reveals the presence of p53 protein (original magnification ×100). **B,** Individual exons of the p53 gene are subjected to PCR followed by size separation by agarose gel electrophoresis. Here, individual exons are seen as single bands. **C,** An exon is then sequenced by direct DNA sequencing by using an automated DNA sequencer. The steps are similar to manual sequencing; however, the nucleotide sequence, as shown in the *top lane*, is seen as *colored peaks* rather than bands on a gel. Below are the normal and tumor p53 sequences. There is a mutation of a G for an A in the tumor (an *arrow* highlights an *R*, indicating a mutation in the sequence trace).

complementary DNA or RNA probes followed by detection of the label on an x-ray film by autoradiography (the use of radioactivity to excite photographic emulsion; applied to the detection of gene expression and cell kinetics in tissues) or by chemiluminescence (the emission of light as a product of a chemical reaction).

*Northern and Western blotting.* A modification of Southern blot analysis permits study of RNA from tissues. By analogy to Southern blotting, this method was referred to—initially in a jocular context—as *Northern blotting* (Table I).[50] The term is widely accepted now. The separation and identification of proteins in a similar fashion is referred to as *Western blotting*. The phrase *Eastern blotting* has not, to date, been attached to a laboratory technique.

Northern blotting consists of RNA size-separated by means of agarose gel electrophoresis, transfer to nylon or nitrocellulose membrane, and hybridization with specific, labeled DNA or RNA probes. RNA is sensitive to degradation by heat-stable ribonucleases that resist common sterilization methods. Single- stranded RNA tends to stabilize by folding into double-stranded configurations termed *hairpin loops*, distorting the RNA and interfering with its analysis. To prevent these changes in RNA during analysis, separation gels must be run in the presence of strong denaturing agents such as formaldehyde or methyl mercury. Laser densitometric scanning of the signal on blots obtained with DNA or RNA extracted from tumors provides a means of quantitative analysis of oncogenes. However, a limitation of these types of investigations is that the genetic material is removed from its topographic surroundings. The genetic material under investigation comes from a heterogenous collection of stromal and neoplastic cells. This "contamination" with stromal cells dilutes the signal from the neoplastic cells.

*ISH.* ISH is a technique used to examine DNA and RNA in their normal topographic surroundings. The technical approaches to the identification of DNA and RNA differ slightly but are conceptually similar to blotting techniques previously described, whereby a

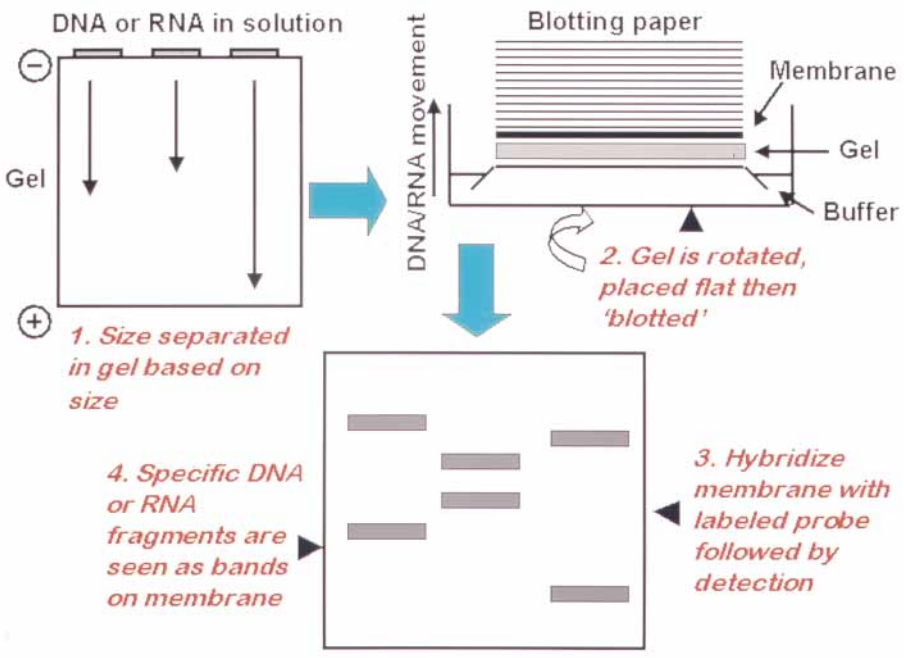


Fig 7. Blotting methods involve size separation by gel electrophoresis of DNA or RNA that has been previously cut by restriction enzymes. The smallest fragments migrate the farthest from a negatively charged electrode. Next, a buffer migrates from a well, through the gel and overlying nylon membrane, and into blotting paper. The blotting paper functions to draw up the buffer and DNA/RNA in a capillary fashion into the membrane. The genetic fragments in the gel are then immobilized in the membrane. The membrane is removed and hybridized with labeled DNA or RNA probes that demonstrate the specific bands of genetic material that are present.

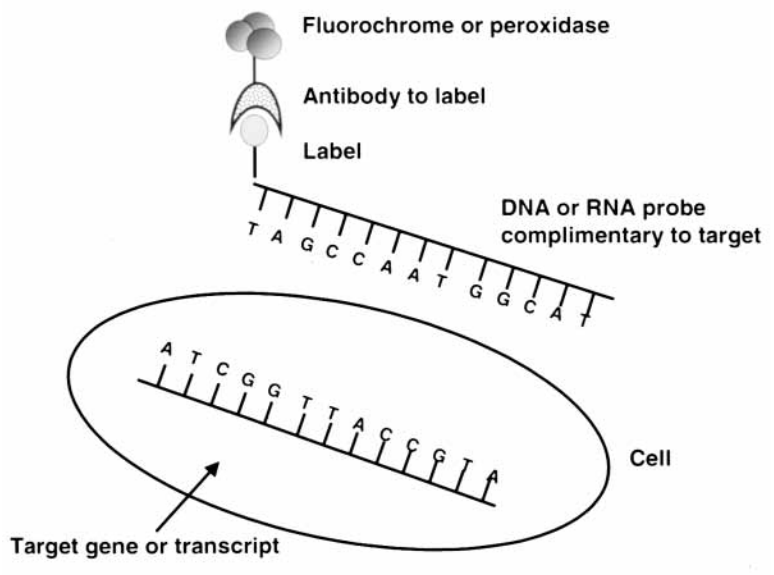


Fig 8. Principles of in situ hybridization. DNA or RNA in a cell is identified in situ by using a complementary probe of RNA or DNA. The labeled probe is detected by using an antibody directed against the label. The complex is then visualized by means of a fluorochrome or by peroxidase reaction of a substrate, similar to immunohistochemistry.

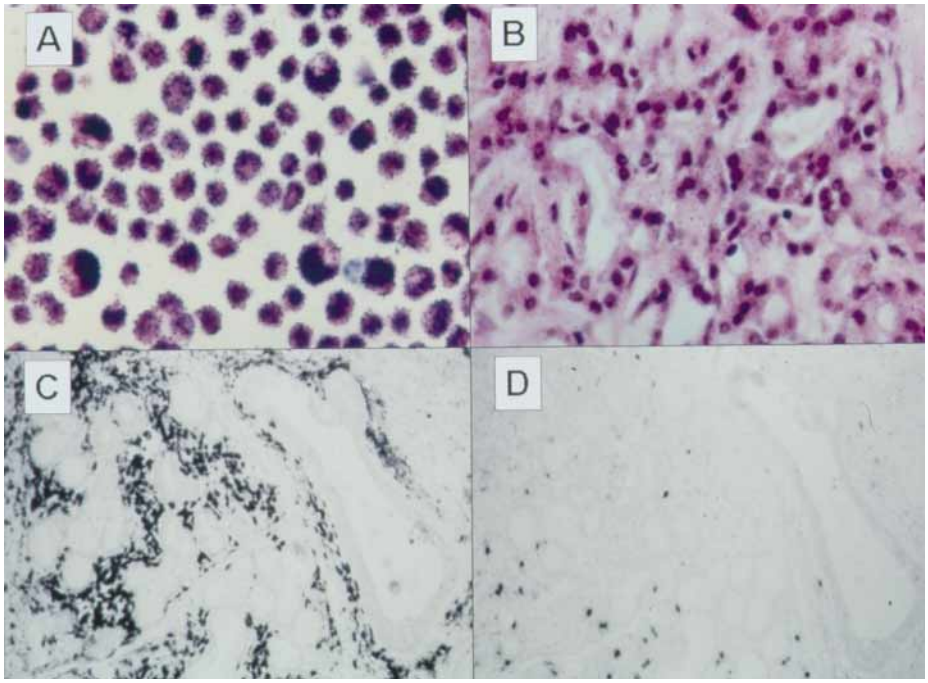


Fig 9. In situ hybridization (ISH) is used to demonstrate DNA or RNA in cells and tissue sections. **A,** The demonstration of *HER2/neu (c-erbB-2)* mRNA expression in the breast carcinoma cell line MB-MDA-231 is known to overexpress the gene. An mRNA probe has been labeled with radioactive $^{35}$-S, and each signal (*black dot*) represents *HER2/neu* mRNA (original magnification ×160). **B,** *HER-2/neu mRNA* overexpression in an acinic cell carcinoma (original magnification ×160). **C,** Kappa light-chain restriction demonstrated in a labial salivary gland biopsy by nonisotopic ISH. Almost all the lymphocytes and plasma cells express kappa light-chain mRNA (original magnification ×25). **D,** The same gland shows little lambda light-chain mRNA expression (original magnification ×25). These findings indicate that the infiltrate is light chain–restricted and, therefore, monoclonal, supporting a diagnosis of lymphoma. C & D are reproduced[63] with permission from Wiley Publishers (John Wiley & Sons, Inc.).

labeled probe is used to hybridize with target nucleic acids (Fig 8).

ISH, using radioactive labeled probes, was first described in 1969.[51,52] Recombinant DNA technology and isotopic labeling procedures permit demonstration of single-copy genes in metaphase (the mitotic phase in which the condensed chromosomes are attached to the spindle fibers and line up in the middle of the cell) chromosome spreads known as fluorescence in situ hybridization (FISH; discussed in "Other advanced technologies"). Subsequent developments of ISH include the use of nonradioactive probe labels, which have improved procedure safety and simplicity, closely approaching the sensitivity levels of techniques using radioactive labels.

ISH has a wide range of applications in pathology. It has been used to detect Epstein-Barr viral particles in oral hairy leukoplakia, a lesion often seen in immunosuppressed individuals such as those infected with the human immunodeficiency virus.[53,54] ISH has found other applications in microbiology, embryology, cytogenetics, and neurobiology. Applications of ISH include the study of homeotic gene expression (ie, "master switch" genes, some of them oncogenes) during embryogenesis, of the role of genetic "memory" (sexual imprinting by methylation of DNA) in gene expression, and of the effects of neuroendocrine stimuli on gene expression in neurons.[55]

Traditionally, viruses have been identified by culture techniques. Although this process is sensitive, it is technically difficult, requires significant time, and is not applicable to all types of viruses. ISH and immunohistochemistry have provided new methods for the identification and study of many viruses. Although ISH analysis for detection of viral DNA and immunohistochemical analysis for identification of viral proteins provide equal sensitivity in disclosing evidence of virus, the former technique is often preferable when no antigen is present in the tissue section or when commercially prepared antisera are unavailable.

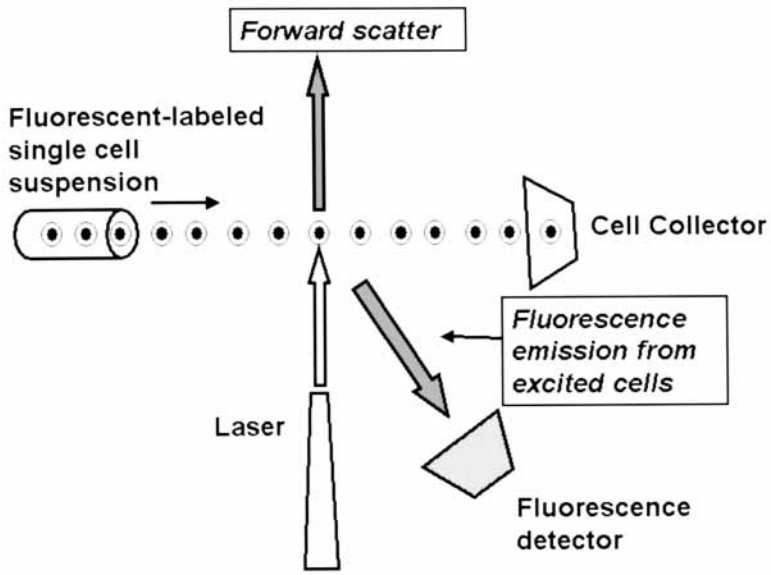


Fig 10. The principle of flow cytometry and cell sorting. Single cells are stained with a fluorescent dye whose incorporation is proportional to the amount of DNA present. The cells are then passed along a charged column through a laser beam. The amount of fluorescence is measured to determine the proportion of cells in different phases of the cell cycle, and the amount of forward scatter measures cell volume. The use of labeled antibodies also permits the determination of the expression profile of different proteins in a cell.

ISH has been used to identify cytomegalovirus infection in both the retina and the lung[56] and to identify hepatitis B virus in hepatocytes.[57] Human papillomavirus has been associated with a variety of benign and malignant epithelial lesions, most notably occurring in the uterine cervix. ISH has been used to identify, retrospectively, many subtypes of human papillomavirus associated with cervical and vulvovaginal lesions[58] and oral verruciform lesions.[59,60] Work from our group has shown the utility of ISH for the study of head and neck neoplasia by characterizing *c-erbB-2 (HER2/neu)* overexpression in salivary gland tumors (Fig 9, *A* and *B*)[61] and for the study of clonality in lymphocyte populations of occult marginal zone lymphoma (Fig 9, *C* and *D*).[62,63]

## Other advanced technologies

*Flow cytometry.* Flow cytometry is an important method used to analyze cell kinetics (the distribution of cells in different phases of the cell cycle) and protein expression in normal and tumor cells. For the determination of cell kinetics, flow cytometry offers many advantages over autoradiography, including speed and automation. In addition, large numbers of cells can be analyzed rapidly, providing a distribution profile of several thousands of cells at one time.

Flow cytometry permits analysis of tissue when prepared as a single-cell suspension stained with a DNA-binding fluorescent dye. The amount of fluorescence thereby corresponds to the amount of DNA present in the cell. The labeled cells are then directed, in single file along a charged column through a laser beam, which excites the fluorescent dye bound to the cell. The fluorescent emissions from the excited cells are then collected by a fluorescence detector and analyzed. Cell size can also be detected by using data from the forward scatter of the excitation laser passing through the stream of single cells (Fig 10).

A number of different dyes have been used in flow cytometry, including ethidium bromide (a dye that stains DNA orange when viewed under an ultraviolet light), propidium iodide, acridine orange, mithramycin, and Hoechst 33342. Acridine orange is particularly useful because it permits the separation of cells based on the amount of double-stranded DNA (green fluorescence) and single-stranded RNA (red fluorescence). Most dyes require fixation of the cells, but some dyes such as Hoechst 33342 bind to viable cells. Fluorescence-labeled antibodies can also be used that bind to specific cellular proteins, which can be detected when the cells pass through the laser.

Flow cytometry can be used to provide information on the distribution of cells in the cell cycle based on the proportion with 2N DNA (G0 and G1), 4N DNA (G2

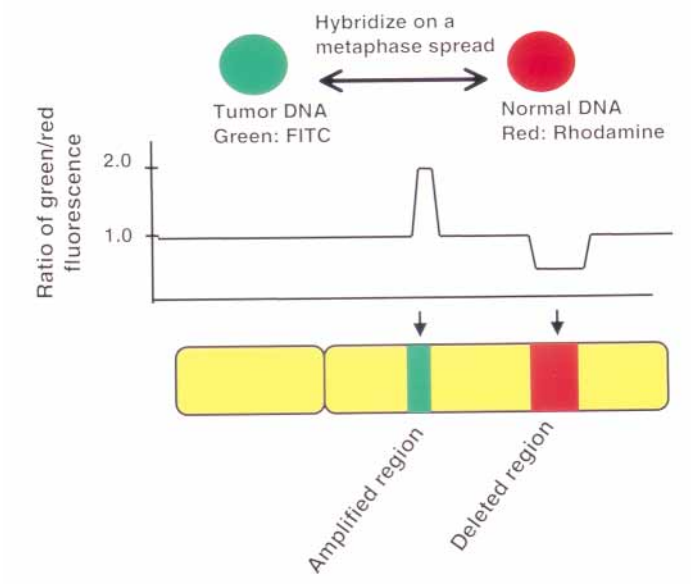


Fig 11. Comparative genomic hybridization is an in situ method to detect regions of gene loss or gain. Tumor DNA is labeled with fluorescein isothiocyanate (FITC) to produce green fluorescence, and normal DNA is labeled with rhodamine to produce red fluorescence. Both tumor and normal DNA are then hybridized onto normal metaphase chromosomal spreads. Amplified regions of DNA are detected when the ratio of green to red fluorescence is greater than 1, resulting in green fluorescence. Regions of chromosomal loss are detected when the green/red ratio is less than 1, resulting in red fluorescence. Used with permission from Squire J, Philips RA.[48]

and M phases), and intermediate DNA content (S-phase). Computer modeling can then generate a profile of all the cells and the proportion in each phase of the cell cycle. The technique can also be applied to the kinetic study of cells obtained from paraffin blocks. Hedley et al,[64] in 1983, described a technique wherein thick (ie, 30 μm) sections are cut from tissue blocks and single-cell suspensions are prepared by incubation of sections in a proteolytic solution, and then the cells are stained. This method is particularly useful for the retrospective study of cell kinetics in archival material such as can be obtained from tumor banks.[65]

Because single-cell suspensions are a prerequisite for flow cytometry, lymphomas and leukemias have lent themselves particularly well to this technology. These cell populations lack intercellular adhesion molecules and can be easily separated into single-cell suspensions. Flow cytometry has been used to define lymphoma and leukemia subtypes and to separate lymphomas from forms of reactive lymphoid hyperplasia. B-cell lymphoma is monoclonal and expresses only one type of immunoglobulin light chain, either kappa (κ) or lambda (λ). Therefore, lymphocyte population expression of a mixture of κ and λ light chains is strongly suggestive of reactive—rather than neoplastic—cell proliferation. By contrast, a population of lymphoid cells that expresses only κ or λ (light chain restricted) is deemed monoclonal and is more likely to represent neoplastic disease. Flow cytometry is very sensitive and can detect a monoclonal population of lymphocytes constituting only 5% to 10% of an otherwise polyclonal population of cells.[66] Flow cytometry can also be used to define the expression profiles of proteins in tumor cells, including growth factors, protein products of oncogenes, and markers of drug resistance such as P-glycoprotein.[67]

*FISH.* Fluorescence in situ hybridization has been used to map genes on chromosomes and to characterize chromosomal abnormalities. Although conceptually identical to in situ hybridization, which was described earlier, it differs in several aspects. A DNA probe, labeled with biotin or digoxigenin, specific for a chromosome segment or a whole chromosome is used. Chromosomes are typically studied in metaphase spread with warming to 75°C in 70% formamide to denature the DNA into single strands. The DNA probe is then hybridized to chromosomes. After nonspecifically bound probe is washed off, the section is incubated with a fluorescence-labeled *antibody* directed against biotin or digoxigenin.[68]

FISH can be used to order genes and DNA segments on chromosomes to a resolution of 2 to 3 megabases. On the basis of the amount of fluorescence, FISH can be used to determine gene amplification or loss, although

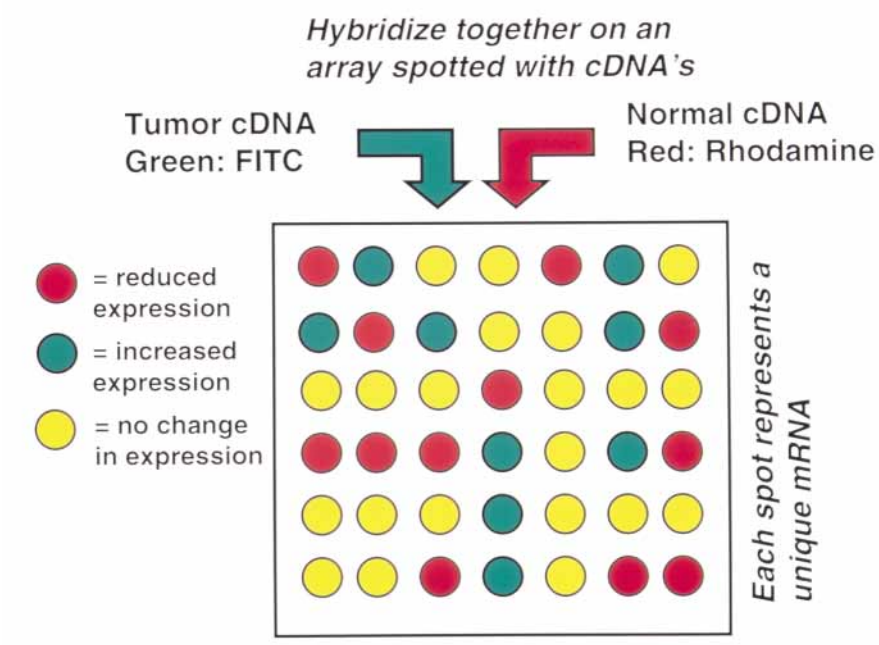


Fig 12. The analysis of gene expression by cDNA microarrays involved the hybridization of labeled tumor and normal cDNA onto a "chip" that was spotted with known cDNA. Reduced gene expression is seen as an increase in red, whereas increased gene expression is seen as a green color. No change in expression above normal levels is seen as yellow. Computer quantification is often needed because subtle changes in color may not be detectable with the naked eye.

the resolution is not high. FISH can also be performed on cells in interphase (the phase of mitosis in which the chromosomes are condensed), where this technique is useful for the determination of gene amplification by multiple gene copies and numerical changes in chromosomes. Here, FISH is usually combined with confocal microscopy, a computer-assisted imaging method to examine thin serial sections of whole cells in interphase. Chromosome *deletions,* translocations, and breakpoints can also be detected by using FISH. The *bcr-abl* rearrangement and *CCND1* (cyclin D1) gene amplification can be rapidly determined using FISH.[41,69]

*Comparative genomic hybridization.* When the cytogenetic abnormality is unknown, a suitable probe for FISH cannot be selected. Comparative genomic hybridization, first described by Kallioniemi et al,[70] permits the development of a detailed map of chromosomal differences between normal and tumor cells by detecting increases (amplifications) or decreases (deletions) of segments of DNA. The technique involves labeling tumor DNA with biotin, which is detected with fluorescein (green), and labeling normal DNA with digoxigenin, which is detected with rhodamine (red; Fig 11). DNA samples from both normal and tumor are then hybridized together onto a metaphase spread of unlabeled normal chromosomes. Regions of gain or loss of DNA such as deletions, duplications, or amplifications are seen as changes in the relative ratios of red and green. Thus, areas of amplification are represented by green and areas of loss by red. Subtle changes in color may not be discernible by the naked eye, requiring sophisticated image analysis software for quantification of these gene regions.

A major disadvantage of comparative genomic hybridization is its relative insensitivity, in that only chromosomal changes larger than 5 megabases can be detected. However, the technique is useful for the identification of relatively small chromosome translocations that cannot be detected by traditional Geimsa staining of metaphase spreads (karyotyping) and for the identification of novel gene amplification or loss in tumors.[71] Balanced rearrangements such as inversions and translocations cannot be detected by comparative genomic hybridization.[72]

*Microarrays.* A major advance in the quantitative study of mRNA expression has been the development of microarray technology, commonly referred to as "DNA chips." By using microarrays, the expression levels of hundreds to thousands of genes can be determined at the same time, providing a unique profile of

Fig 13. Diagram representing the principle of laser capture microdissection (LCM) and the main components of the LCM apparatus. Modified from Fend and Raffeld.[83] Reproduced with permission from the BMJ Publishing Group.

increased or decreased gene expression in tissues. Although an emerging technology, molecular expression profiles have been examined in forms of lymphoma and melanoma, with findings suggesting alternative taxonomy on the basis of molecular differences in the expression of novel genes.[73,74]

Conceptually, DNA microarray technology (Fig 12) is similar to the underlying principles of Northern and Southern blotting. The process relies on the hybridization of a "probe" to multiple defined genomic DNAs, cDNAs, expressed sequence tags, or oligonucleotides that have been "printed" onto specific locations of a solid phase or "chip."[75] The probe is usually cDNA fragments produced by reverse transcription of tumor mRNA and then labeled with a fluorescent marker. The probe and the spotted cDNAs are hybridized, resulting in varying red/green and yellow fluorescent emissions. These emissions are then scanned by a reader consisting of argon lasers and a scanning fluorescence confocal microscope. The use of computer image analysis permits quantification of the intensity of thousands of different genes on the array, which can then be compared to the expression in normal tissues. The instrumentation required to perform microarray analysis consists of an arrayer to spot the cDNAs onto a chip, a reader with laser to detect and measure the fluorescent emissions, and a computer imaging system to record and analyze the data. Although microarray analysis can be obtained through commercial sources, many research laboratories are assemblying their own technologies for testing.[76]

Because this is an evolving technology, the scope of applications has yet to be defined. Some of the types of studies that have used microarray technology include gene expression studies in tumors versus normal tissues,[77] genotyping of mutations in tumors, functional analyses of genes expressed in yeast,[78] and gene mapping to identify loci of disease susceptibility genes.[79]

*Laser capture microdissection.* The molecular analytical techniques described in the preceding sections have developed rapidly in recent years and are now culminating in many applications in pathology, most recently those involving DNA microarrays. When used to study the molecular fundamentals of pathologic processes, these approaches offer exciting opportunities to extend our understanding of disease. However, one major limitation of the application of molecular

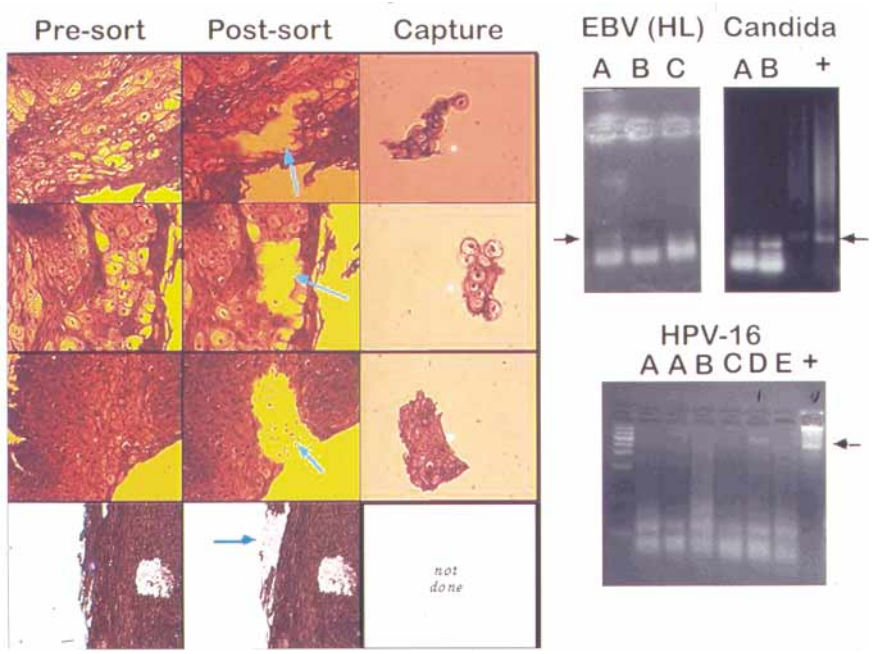


Fig 14. LCM sorting of biopsies followed by PCR analysis. The images on the left show sections from a biopsy of hairy leukoplakia (*HL), top three rows*, or pseudomembranous candidiasis (*bottom row*) before sorting (*left column*), immediately after sorting (*middle column*; *blue arrows* identify the cells removed by LCM), and the cells that have been captured (*right column*) before deposition into a tube for molecular analysis. *On the right* are the PCR products specific for each pathogen. *(EBV) Lanes A-C* are derived from separate sorts; the PCR product is visible in lane A, barely detectable in lane B. *Lane C* is a high dilution of a positive control cell line (Raji). Amplification is of a 150 base-pair segment of BMRF-2 gene. (*Candida*) *Lanes A and B* are separate sorts; + is *Candida* from culture. Amplification of a 300 base-pair segment from *Candida* ferric reductase–like protein is visible in all 3 lanes. Amplification of DNA from cells selected for HL analysis were negative (not shown). (HPV-16) Of 5 different sorts (*A* was PCR-amplified twice), a 400 base-pair HPB L-1 sequence was detectable in 1 (*D*). The *Candida* and HPV samples are from paraffin-embedded tissue; the HL sample is from a frozen biopsy. The *Candida* and HPV samples are courtesy of Dr Mario Roederer.

biology to pathology has been the heterogeneous nature of available tissue samples. Tissue samples of tumors, inflammatory lesions, infections, and even normal tissues consist of complex, heterogeneous mixtures of cells. To develop useful, reproducible data, it is desirable to study pure populations of cells obtained from actual biopsy or autopsy samples of tissues, both normal and diseased.

Techniques available to approach this problem have included animal studies, xenografts, cell cultures, flow cytometry, enzyme separation of tissue layers, and microdissection. Each has significant limitations. Animal models offer an approximation—at best—of human conditions. Moreover, the problem of tissue heterogeneity persists in these models. Cultured tumor cell populations, although providing a relatively homogeneous population of cells, often diverge in many characteristics from their cells of origin, even when the latter are known with certainty. Enzyme separation, as is applicable, for example, to sheets of skin or mucosal epithelium, is limited to a small number of anatomic locations. Flow cytometry has been an invaluable method, permitting the study of specific cell populations in suspension obtained from cell mixtures found within tissues. However, relating the results of suspended cells back to their in vivo tissue origin and location is difficult, and putatively specific markers are required. For these and other reasons, a number of microdissection approaches have been attempted. These include needle scraping of cells from sections and negative ablation, in which the cells of interest are covered with light-absorbing pigment and the rest of the specimen, or at least its DNA, is destroyed with ultraviolet light. Both strategies are technically challenging and have significant limitations. Thus, the recent development of laser-based methods for microdissection has been a significant advancement in the field.

Laser capture microdissection (LCM) is a new and

exciting technology for rapidly preparing relatively pure cell samples from tissue sections. LCM was developed at the National Cancer Institute laboratories on the National Institutes of Health campus (Bethesda, Md, USA).[80] A number of excellent recent reviews are available.[81-83] In large part, the original impetus for this technology appears to have been the need to develop expression libraries (ie, collections of mRNA from defined cell populations) from malignant and premalignant lesions. At least 2 different devices are now available commercially.

The principle on which LCM is based is the preferential adherence of identified cells to a plastic membrane activated by a low-energy infrared laser pulse (Fig 13). The full apparatus consists of the following: (1) inverted microscope, (2) infrared laser diode, (3) laser controls, (4) microscope stage controlled by joysticks, (5) slide immobilizer by vacuum, (6) charged-couple device camera, (7) color monitor, and (8) thermoplastic membrane for cell transfer—approximately 6 mm in diameter, mounted on an optically clear cap that fits on standard 0.5-mL microcentrifuge tubes for further analysis.

The process is summarized in Fig 13 and illustrated in Fig 14. As illustrated, the PixCell device (Arcturus Engineering, Mountain View Calif, USA) has a mechanical transport arm, on which is suspended the cap, which is placed on the area of interest within a dehydrated section. The cells to be studied are visualized through a microscope, and the area is selected by using a positioning beam. Then, laser activation is initiated and focal melting of the plastic membrane follows. The cells adhere to the membrane rather than to the glass slide and can then be lifted by raising the cap. The adherent cells are then transferred to a microcentrifuge tube containing appropriate reagents and buffers. The low energy levels of the laser result in a modest temperature increase that does not degrade the DNA, RNA, or proteins of interest.

The method is fast, precise, and adaptable to a wide range of tissues and molecules to be studied. Both the tissue left behind and the tissue retrieved can be identified, and the morphology of both is excellent. Large numbers of well-characterized cells can be obtained within a few minutes. New operators can learn the method rapidly, although histologic expertise is extremely important. Both fresh and archived material may be used, although results from stained tissue sections can be challenging to—and at times impossible to—interpret. The minimum laser spot size is approximately 7.5 μm, rendering isolation of single cells difficult—although not impossible. There is also the risk of contamination from adjacent cells, even with careful laser microdissecting or during transfer of tissues to the microcentrifuge tube.

The related technique of laser pressure catapulting, used in the P.A.L.M. (Positioning and Ablation with Laser Microbeams) device (Mikrolaser Technologie, Bernried, Germany) is another valuable method for cell isolation. In our hands, the P.A.L.M. device has been somewhat slower and more difficult to learn, but it offers advantages when samples as small as single cells are required. The ideal arrangement would probably involve access to both types of equipment.

The applications of microdissection in pathology are legion. A few examples include obtaining pure cell populations from fresh, frozen, or fixed tissues and cytology samples for DNA molecular genetic analysis[84]; gene expression studies involving mRNA such as RT-PCR[85]; and combined immunohistochemistry or immunofluorescence to better identify cells for mRNA analysis.[86] Other studies have combined immunofluorescence studies with flow cytometry, for example, to analyze cell cycle characteristics of nuclei from paraffin sections.[87] In oral disease investigations, LCM has been used to identify immunoglobulin genes in plasma cells in salivary glands[88] and to study the expression of differentiation and growth-related genes in oral cancer.[89,90]

Recently, our group has used LCM to isolate the DNA from relatively pure populations of preneoplastic and neoplastic oral epithelial cells, from routinely processed biopsies, providing new insights into p53 gene mutations,[29] amplification of the *cyclin D1* gene, and loss of heterozygosity[91] (loss of one allele of a gene when both alleles are not identical) in oral cancer development.[39] LCM has also permitted us to characterize homozygous deletions (loss of both alleles of a gene; a mechanism to inactivate some tumor suppressor genes) of component exons of the *CDKN2A* gene, which encodes for the $p16^{INK4A}$ protein in sequential oral dysplasias and carcinomas.[92] These genetic alterations would be undetectable in a mixed population of normal and tumor cells.

The LCM approach is only 4 years old, and application to oral pathology is, as yet, rare. However, it seems reasonable to characterize this approach as one of the more exciting developments in investigative pathology in a generation—and one that has the potential to help answer many outstanding questions in our field.

## SUMMARY

Advances in the analysis of DNA, RNA, and proteins are currently revolutionizing the practice of surgical pathology. These changes have permitted improved insights into a range of disorders and have led to improvements in the understanding and classification of many diseases. This article has described many of the most important molecular, immunohistochemical,

and immunofluorescent tools that are currently being used or will be used in diagnostic pathology, as well as their applications. In the future, the pathologist will continue to play a central role in diagnosis and it is conceivable that the armamentarium of diagnostic tests will include many of these advances. Thus, these changes likely will improve the understanding of diseases that affect the head and neck and the ability of the pathologist to render a diagnosis.

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