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Analysis of Genes, Transcripts, and Proteins via DNA Ligation

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Abstract

Analytical reactions in which short DNA strands are used in combination with DNA ligases have proven useful for measuring, decoding, and locating most classes of macromolecules. Given the need to accumulate large amounts of precise molecular information from biological systems in research and in diagnostics, ligation reactions will continue to offer valuable strategies for advanced analytical reactions. Here, we provide a basis for further development of methods by reviewing the history of analytical ligation reactions, discussing the properties of ligation reactions that render them suitable for engineering novel assays, describing a wide range of successful ligase-based assays, and briefly considering future directions.

1. INTRODUCTION

PCR: polymerase chain reaction

Rolling-circle amplification (RCA): amplification reaction for circular DNA that results in concatemeric products

Progress in molecular biology crucially depends on the tools available to investigate biological processes. In this regard, it is striking that the combination of synthetic DNA strands and a repertoire of enzymes acting on these strands has yielded numerous powerful and widely used tools. For example, the successful use of DNA polymerases with oligonucleotides has resulted in two Nobel Prize–awarded methods: Sanger sequencing (1) and the polymerase chain reaction (PCR) (2).

The combination of synthetic oligonucleotides with DNA ligases—the subject of the present review—has also resulted in a remarkable range of molecular tools, and the technology has the potential to spawn new generations of methods to meet future analytical needs. The act of joining two DNA strands, hybridized next to each other on a target nucleic acid sequence, has proven useful in analyses serving to (*a*) measure, (*b*) determine the sequence composition of, and (*c*) image the distribution of biomolecules. Some of these assays have become powerful research tools, others are now being applied for routine diagnostic purposes, and still others are only now emerging to find their place in research and diagnostics. Nonetheless, molecular analyses far more effective than those currently available are required to meet present and future analytical challenges. Examples of such challenges include systems biologists' need to capture global information about molecular states of cells and tissues to model the behavior of biological systems and the need to acquire global information about genomes, transcriptomes, and proteomes in large collections of patient samples to identify biomarkers and drug targets useful for diagnostics and therapy and to reveal disease mechanisms. Ligase-based assays will continue to play important roles in all these kinds of analyses.

Several features of ligase-based assays help explain their popularity.

1. The dependence of target sequence detection on two recognition events by pairs of probes to be joined by ligation ensures high specificity, as spurious hybridization by one or the other detection reagent fails to give rise to the diagnostic ligation reaction.
2. The requirements for substrate recognition by the ligase can assist in distinguishing similar target molecules. Even a single mismatched base pair near the site of ligation strongly inhibits the ligation reaction. This property is the basis for ligase-assisted genotyping and DNA sequencing procedures.
3. The fact that ligation reactions give rise to integral DNA strands means that an efficient, kinetically favored hybridization reaction matures into a covalently bonded reaction product. DNA ligation reactions can therefore be driven to completion due to the irreversible nature of the product formation. The covalent bond provides a very strong link that is resistant to even highly stringent washing conditions. This mechanism has proven useful in both analytical and preparative applications.
4. The new DNA strands that form by ligation can be subjected to numerous techniques for copying. These techniques can amplify ligated products to large numbers, whereas unligated reagents remain inert.
5. Notably, the amplified DNA strands transmit information that has been encoded in the probes. This is the basis for many multiplexed assays in which probe sets designed to interrogate many different target molecules in the same sample can be separately identified and recorded after joint amplification.
6. In a particular type of ligase reaction, the ends of DNA probes are joined to form circular strands. This has several valuable consequences, such as the opportunity to remove excess probes through use of exonucleases to digest DNA strands with free ends while keeping successfully circularized molecules intact. The circularized molecules are also suitable substrates for amplification via rolling-circle amplification (RCA). This technique yields

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large single-stranded DNA molecules, each composed of hundreds of complements of the probes. In contrast, unreacted probes fail to give rise to any such products. The mechanism of probe circularization is a common element in several of the assays that we describe below.

In this review, we describe some fundamental aspects of ligase-based analytical reactions and briefly review some of the more popular methods. We also discuss in detail the methods developed in our lab. Finally, we conclude with a perspective toward future developments of ligase-based analytical reactions.

SNP: single nucleotide polymorphism

OLA: oligonucleotide ligation assay

LCR: ligase chain reaction

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2. A HISTORY OF DNA LIGATION ASSAYS

The Nobel Prize winner H.G. Khorana and his team pioneered the field of ligase-based DNA detection in the early 1970s (3). They used oligonucleotide ligation with T4 DNA ligase to analyze a transfer RNA gene and showed that proximity between the hybridization sites of two oligonucleotides was necessary for their successful ligation.

The ability of DNA ligases to discriminate single mismatched base pairs was exploited early on by several investigators to detect single nucleotide polymorphisms (SNPs) and point mutations. Landegren et al. (4) developed the oligonucleotide ligation assay (OLA) and demonstrated its suitability for distinguishing the SNP that causes sickle cell disease from the normal human *ß globulin* gene in either cloned or amplified DNA, or directly in total human genomic DNA. They used two 20-nt-long synthetic oligonucleotide probes that hybridized to adjacent sites on the target DNA sequence (4). Two alternate probes had 3′ nucleotides specific for the allelic forms of the investigated gene, and these were combined with one invariant probe hybridizing downstream of either of the other two. The authors detected SNPs located at the junction of the two probes by using T4 DNA ligase to covalently join the pairs of probes. Successful ligation was detected by gel separation or by labeling one probe with biotin for capture on a solid phase and labeling the other with a fluorophore or a radiolabel. All of the 12 possible mismatched base pairs between probes and targets were shown to inhibit ligation under conditions where all four matches were efficiently ligated (4).

Alves & Carr (5) devised a method similar to OLA to detect point mutations in the Ha-ras oncogene. The melting temperature of two oligonucleotide probes was increased by template-dependent ligation upon recognition of the target sequence, allowing nonspecifically hybridized probes to be removed by stringent washes. This method reduced nonspecific background such that simple dot-blot assays could be carried out to detect SNPs in total human genomic DNA without prior amplification.

Wu & Wallace (6) further developed the SNP-detection system, increasing the sensitivity by adding an amplification step. Their ligase-amplification reaction (LAR) uses pairs of probes for both strands of the target sequence. Thermocycling of the reactions serves to separate ligation products from target sequences, and it allows newly formed ligation products to template new ligation reactions in succeeding cycles, leading to exponential amplification. However, this reaction would be more efficient and practical with a thermostable ligase.

In 1991, Barany (7) described the successful isolation, cloning, and expression of a thermostable ligase from *Thermus aquaticus*. This thermostable ligase was used in the ligase chain reaction (LCR), an improved version of LAR, eliminating the need to replenish ligase after every cycle. Raising the reaction temperature closer to the melting temperatures of the oligonucleotides allowed for more specific hybridization, and it also eliminated target-independent blunt-end ligations (8).

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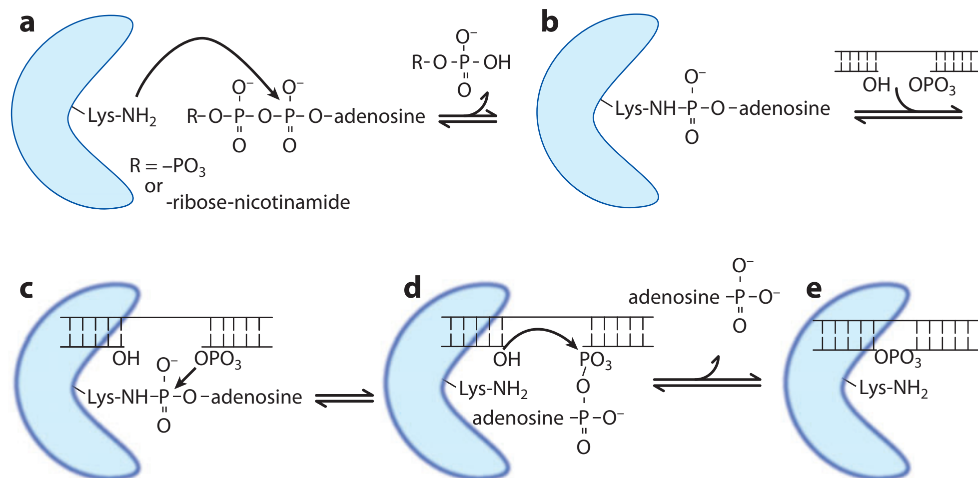

Figure 1

Mechanism of enzymatic DNA ligation. First, a primary amino group at the active site of the ligase is adenylated, preserving the energy of a phosphodiester bond from either NAD^+ or ATP, depending on whether the enzyme is of eubacterial or archaeal origin, respectively (*a*, *b*). Upon binding to a nicked DNA duplex, this adenylyl group is transferred to the 5′ phosphate at the nicked site (*c*). Finally, the enzyme catalyzes an attack of the activated 5′ end by the nearby 3′-hydroxyl group (*d*), resulting in the formation of a phosphodiester bond between the 5′ and 3′ ends and the release of AMP (*e*).

3. THE ENZYMOLOGY OF DNA LIGATION

DNA ligases—the enzymes used in all the assays discussed in this review—are a group of enzymes that catalyze the joining of two juxtaposed ends of DNA strands. The two strands to be joined must bear a terminal 5′-phosphate and a 3′-hydroxy group, respectively, and the formation of a phosphodiester bond requires energy supplied from the phosphodiester bond of either ATP or NAD^+ (**Figure 1**) (9–11). The enzymes are normally involved in closing nicks—breaks in single strands in duplex DNA—during DNA replication, recombination, and repair reactions. The ligases' usefulness in biotechnology rapidly became apparent after the first biochemical characterization of T4 DNA ligase in 1967 (9). DNA ligases have been isolated from numerous organisms and have been characterized for their ability to meet particular assay requirements (4, 13–26). T4 DNA ligase remains the most commonly used DNA ligase in molecular assays. This 68-kDa monomeric protein from bacteriophage T4 uses ATP as an energy source, as do DNA ligases from most other organisms. However, eubacterial ligases use NAD^+ to drive the DNA joining reaction. Highly useful DNA ligases have been isolated from thermophilic organisms and are used at higher reaction temperatures, where oligonucleotides are less likely to interact stably with mismatched target sequences, thereby improving selectivity of detection. They can also be used in reactions where the temperature is varied cyclically to permit denaturation, hybridization, and ligation (27).

Similarities between the crystal structures of T7 DNA ligase with bound ATP cofactor and the *Chlorella* virus mRNA-capping enzyme with bound GTP suggested a common core structure and shared enzymatic mechanisms and thereby provided insights into the molecular basis for substrate recognition by ligases (28, 29). This information helped identify domains important for the steps of the ligation process (30–32). Studies of substrate recognition by DNA ligases further revealed that *Chlorella* and T7 DNA ligases both have asymmetric footprints across the nick extending 8–9 nt and 3–5 nt, respectively, on the 3′ side of the nick and 11–12 nt and 7–9 nt,

respectively, on the 5′ side (33, 34). The sizes of the different enzymes' footprints affect the design of ligation probes. Mismatches in positions covered by the footprints of ligases significantly reduce the efficiency of ligation and contribute to the sequence selectivity of assays. However, enzymes generally discriminate most strongly against mismatches near the nick site. Knowledge of the position-dependent selectivity of each ligase should guide the design of oligonucleotide probes. For instance, both T4 DNA ligases and *Tth* DNA ligases have been shown to discriminate more strongly against mismatches at the 3′ end at the nick than at the 5′ end, and this may be a general property of DNA ligases (13, 35). Both these DNA ligases exhibit at least a 1000-fold-lower rate of ligation of substrates with a single mismatch at the 3′ end, compared to the corresponding matched substrate (13, 36). The footprint sizes and optimum temperatures of enzymes require a minimum probe length. For example, short probes such as heptamers are not readily ligated by *Tth* ligase, whereas they can be joined by T4 DNA ligase (37).

The fidelity of DNA ligase–catalyzed DNA ligation is affected by buffer composition. T4 DNA ligases, for example, can also join mismatched substrates relatively efficiently at low monovalent-cation concentrations, whereas mismatch ligation reactions are 10,000 times slower than are matched reactions at 250 mM NaCl (36). Additives such as spermine and spermidine also increase the fidelity of T4 DNA ligase–catalyzed reactions (4, 35). The sensitivity to mismatches at the 3′ end can be further increased by deliberately introducing a mismatch at the third position from the ligation site in probes for both alleles, and DNA ligases have been engineered for improved discrimination of mismatches (13).

T4 DNA ligases can join pairs of DNA oligonucleotides hybridized to RNA strands, allowing direct DNA ligase–based RNA sequence analyses (38–42). This reaction is about 1000-fold slower than is DNA-templated DNA ligation, and the concentration of monovalent cations and ATP should be kept low to avoid accumulation of the 5′-adenylated reaction intermediate (**Figure 1*d***), which otherwise is the predominant product of such reactions. The reaction requires an excess of enzyme over substrate, indicating that the enzyme in these cases has a poor turnover or none at all.

4. NONENZYMATIC DNA LIGATION REACTIONS

A number of chemically assisted or autoligation techniques have been developed to join the ends of two adjacent nucleotide sequences in a template-dependent manner. The use of chemical ligation enables joining of oligonucleotides that cannot serve as substrates for enzymatic ligation. As an example, cyanogen bromide preferentially joins the 5′-hydroxyl and 3′-phosphate groups of two adjacent oligonucleotides, in contrast to DNA ligases that join 3′-hydroxyl and 5′-phosphate groups. Chemical ligation is usually less selective for correct base-pairing than is enzymatic ligation (43). For instance, a single-nucleotide mismatch in short oligonucleotides bearing bromoacetamido and phosphorothioate groups resulted in a modest 15-fold-lower yield of ligation products compared to a perfectly matched substrate (44).

In autoligation, the ends of nucleic acid probes are modified so they can be joined covalently in a template-dependent manner, with no requirement for either enzyme catalysis or added chemical reagents. In principle, such modification could enable ligation assays in live cells and tissues under conditions where reactions catalyzed by enzymes or chemical reagents may be difficult. For example, two DNA probes containing a 3′-phosphorothioate end and a 5′-iodothymidine end react to form a phosphorothioate link if they are hybridized next to each other on a template strand, but the reaction rate is much lower compared to ligation reactions catalyzed by DNA ligases. A 180-fold reduction in reaction rate due to single-nucleotide mismatches at the ligation junction has been reported (14), and the discrimination reached $>10^4$-fold when one of the ligation probes

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was short and had one centrally placed mismatch (see Section 5, below). Unlike enzyme-based ligation, the reaction was equally efficient for probes hybridized to both RNA and DNA strands (45).

ASO: allele-specific oligonucleotide

5. ARCHITECTURE OF DNA LIGATION ASSAYS

Ligation reactions can generate longer DNA strands from shorter probes, or they can join the ends of DNA strands to form DNA circles. However, a considerable variety of approaches are used to generate the substrate for ligation in various ligase-based assays (see **Figure 2**). For example, DNA ligation can be used to stabilize the hybridization of an allele-specific oligonucleotide (ASO) probe hybridizing next to a longer probe (46). Here, the sequence selectivity is achieved by the dramatic difference in hybridization stability between a matched ASO and an ASO that includes a centrally placed mismatch to its target sequence (**Figure 2*a***). By joining short probes to longer ones, excellent sequence specificity and stability of hybridization can be achieved. The ASO ligation format was first described by Wallace and colleagues (46). This assay format was used in the

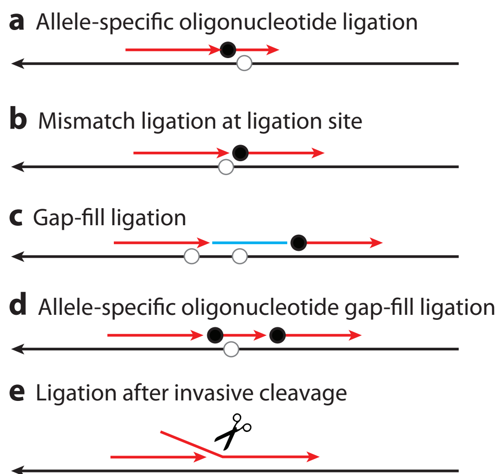

Figure 2

Some ligase assay architectures used for preparative or analytical purposes. Several assay formats are commonly used to quantify and to distinguish target sequences, in particular for genotyping single-nucleotide sequence variants. (*a*) The possibility that a short allele-specific oligonucleotide (ASO) probe ligates to a longer nearby oligonucleotide depends on whether the ASO is correctly base-paired. (*b*) The inherent ability of DNA ligases to discriminate between matched and mismatched substrates at the ligation junction can be utilized to distinguish sequence variants. (*c*) In the gap-fill ligation approach, a target sequence is probed with two oligonucleotides hybridized at a distance from each other. The two oligonucleotides can only be joined by a ligase if the gap is first filled in by a DNA polymerase. Sequence variants can be positioned either at the 3′ end of the upstream oligonucleotide to destabilize DNA polymerization or in the gap, which can then only be filled in the presence of the correct nucleotide. (*d*) This design is similar to that shown in panel *c*, but the gap is filled by ligation to both ends of a short intervening ASO. (*e*) The substrate for a ligation reaction can also be generated by invasive cleavage by a structure-specific endonuclease. The enzyme recognizes the 3′ end of one probe competing for target hybridization with the 5′ end of a downstream probe. After cleavage of the 5′ end, the two hybridized oligonucleotides can be joined by ligation. The 3′ ends of the DNA strands are indicated by arrowheads, and the 5′ phosphates at the ligation sites are represented by filled circles. Open circles indicate positions where a mismatched nucleotide should be placed to maximally inhibit ligation for genotyping purposes.

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autoligation assay described above (14, 45), and it is also applied to sequencing by ligation, as described below.

In the oligonucleotide ligation assay format, the inherent property of DNA ligases to discriminate mismatches located at the ligation junction between two probe molecules is used to distinguish target sequence variants. Here, the diagnostic base in a SNP or a point mutation is typically positioned at the 3′ end of an oligonucleotide to be joined to the 5′ end of a second oligonucleotide (**Figure 2*b***), as first described by Landegren and colleagues (4). The same format is used in the LCR (7), as discussed above, as well as in padlock probe assays and in sequencing by ligation (see below).

Padlock probe: target sequence detection by templated ligation of a single-stranded DNA probe to form a DNA circle

Pairs of probes can be designed to anneal to a target sequence, leaving a gap of one or several nucleotides between them. Upon hybridization, an exonuclease-deficient DNA polymerase is used to fill the gap. When it reaches the nearby 5′ end, a ligase joins the ends (**Figure 2*c***). This gap-fill ligation was first described by Abravaya et al. (47). The procedure is used in the gap-fill LCR, the Golden Gate assay, molecular inversion probe assays, and the gap-fill padlock probe assay (see Section 6.1 for detailed discussion). A gap between two adjacent probes can also be filled using a short ASO, which requires ligation at both ends of the short probe (**Figure 2*d***). Such a gap-fill oligonucleotide ligation approach was described by Lizardi et al. (48).

Another means of generating a DNA substrate resembling a nicked DNA duplex is by invasive cleavage via a structure-specific endonuclease that first recognizes a 3′ end of one probe displacing a 5′ region of another probe from a target strand, then cleaves the 5′ end (**Figure 2*e***). This endonucleolytic activity was first described by Lyamichev et al. (49), and the reaction was subsequently used by Dahl and colleagues (50) to create a ligation substrate.

Padlock probes become joined into single-stranded DNA circles by templated ligation of their 5′ and 3′ ends, thus creating a topological link to long or circular target sequences [hence the name padlock probe (**Figure 3*a***)]. This format was first described by Nilsson and colleagues (16). A mirror version of the padlock probe format is used in the selector probe technique, also described further below, in which a single linear probe molecule hybridizes to both ends of a genomic DNA restriction fragment, such that the genomic fragment is converted to a DNA circle (**Figure 3*b***).

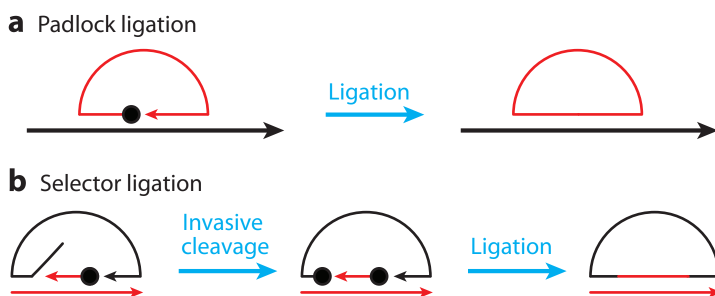

Figure 3

Ligation reactions for DNA analysis through use of padlock and selector probes. Both types of assays are designed to generate circular DNA molecules as a consequence of target sequence recognition. (*a*) In the padlock probe assay, the probe molecule becomes circularized upon binding to a target strand and ligation. Because of the double-helical structure of the hybridized sequence, the ligated padlock probe becomes topologically linked to long target DNA strands. (*b*) The selector probe circularizes a restriction-digested target strand upon binding and ligation. If desired, the 5′ end of the target strand can first be removed by an invasive cleavage reaction to remove unwanted sequences.

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6. APPLICATIONS

6.1. Assays for Genotyping and Copy-Number Measurement

MLPA: multiplex ligation-dependent probe amplification

6.1.1. Oligonucleotide ligation assays. OLAs depend on the target-dependent joining of pairs of oligonucleotide probes. Multiple pairs of ligation probes can be joined in a ligation reaction commonly used to type various loci, and the ligation products can then be amplified by PCR through use of primers hybridizing to common sequence elements at the ends of all the ligated probes (51). A flexible and high-throughput system, developed by Applied Biosystems, allows analysis of 48 SNPs per sample using gel electrophoretic readout. A closely related assay known as multiplex ligation-dependent probe amplification (MLPA) has become a popular way to amplify ~40 target sequences in a single reaction and to analyze their copy numbers by quantifying the amplification products separated by capillary gel electrophoresis (52, 53). MLPA is also used for detecting SNPs, for mRNA profiling, and for DNA methylation analysis following a digestion step.

In the related Golden Gate genotyping assay, multiple SNPs are typed in genomic DNA through a gap-fill ligation approach. The assay uses two allele-specific probes—specific for the SNP in question—and one locus-specific oligonucleotide that hybridizes 1–20 bp downstream of the SNP. By hybridizing further downstream, the oligonucleotide allows for some flexibility in probe design, thereby avoiding potentially challenging sequences adjacent to a SNP site. A polymerase extends correctly (but not incorrectly) base-paired 3′ ends of allele-specific probes, whereupon a ligase joins the extended primer to the downstream probe. Next, all ligation products are amplified using standard PCR primers that are fluorescence labeled to distinguish allele-specific oligonucleotide probes. Each downstream probe contains a unique address tag sequence that directs the capture of amplification products onto a bead array (54–56). This assay has been used in many large-scale genomic projects, and it produced the majority of the genotypes in the ambitious HapMap project, which defined the large-scale genetic landscape of the human genome (57).

Barany and coworkers (58) developed a conceptually interesting ligase-based assay to look for unknown sequence variants. In the first reaction, an endonuclease with a preference for mismatched positions was used to cleave heteroduplexes formed during PCR of DNA from individuals that are heterozygous for sequence variants in the amplified sequence. Next, ligation reactions were performed to repair any erroneously cleaved sites, while DNA cleaved at mismatches resisted joining via the DNA ligase.

6.1.2. Padlock and molecular inversion probe assays. Padlock probes are unimolecular ligation probes that become circularized in a strictly target-dependent manner upon joining of the two target-complementary end sequences (**Figure 3*a***). This circularization approach is well suited for targeted multiplex analysis of genetic variation. The dual recognition of the target sequence provides sufficient specificity to probe unique sequences in the genome (48, 59), and the intramolecular nature of the circularization reaction renders the assays less susceptible to problems with cross-reactivity. In multiplex assays, padlock probes are equipped with tag-sequence motifs in the non-target-complementary segment that joins the target-complementary end sequences. The sequences are used to amplify circularized probes and to sort the amplification product on a microarray comprising complements of the tag sequences (60, 61). Multiplex genotyping assays have been designed using either allele-specific pairs of ligation probes (60) or single probes, leaving out the polymorphic nucleotide position in the probe sequences. In the latter design, a circularization is achieved through a gap-fill reaction, which is performed in separate tubes that contain only one of the nucleotides, and the probes are then cleaved between the target-complementary

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regions to release them from their target sequences prior to amplification with standard primers. Probes of this type are known as molecular inversion probes (MIPs) (61). The MIP technique was developed at the company ParAllele (now part of Affymetrix) to allow 10,000-plex assays (62), and it has been used in a number of large-scale mapping and association studies. The assay is also suitable for analysis of degraded DNA, such as that found in paraffin-embedded formalin-fixed tissue samples, due to the short target sequence for the probes and the fact that the hybridization and ligation reactions are probably more tolerant of DNA damage than are techniques that require target strands to be first amplified by methods such as PCR (63). Wang et al. (63) also demonstrated the technique's ability to detect copy-number variations in a sample.

Circularized padlock probes have also been amplified using RCA (42, 48, 64). In RCA, a circularized probe molecule is replicated over and over again, producing a single DNA strand composed of tandem repeats of complements of the probe molecule. An isothermal exponential hyperbranched RCA (hRCA) can be achieved by adding both forward and reverse primers to the RCA. If a DNA polymerase with strand-displacement activity is used, then primers hybridizing to and extending on the strand produced by RCA displace downstream primer-extension products, creating single-stranded molecules that in turn recruit new primers (42, 48). Padlock probes have been combined with hRCA readout in genotyping assays (65–68).

Exponential amplification of padlock probes can also be achieved using a ligation-driven RCA-based approach termed circle-to-circle amplification (69). In this technique, RCA products (RCPs) are monomerized by restriction-digestion following hybridization to an oligonucleotide. After digestion of the RCPs, the oligonucleotide, which is added in great excess over RCPs, also serves to template circularization of the monomers by ligation. The circles that form can then template a second cycle of RCA, primed from the same oligonucleotide. In each cycle of this process, an amplification factor of 1000 can be achieved.

6.1.3. Selector probes. Selector probes are reagents that can be employed to amplify a large set of arbitrary restriction fragments in multiplex (50). They are designed to hybridize to both ends of single-stranded restriction fragments and to template circularization of these genomic DNA fragments. Also, through use of a standard PCR primer pair, a general vector oligonucleotide containing sequences required for amplification of all selected fragments can be inserted into the circles (**Figure 3*b***). The ligation of the 5′ end of the genomic target sequence can also be achieved after invasive cleavage (as shown in **Figure 2*e***) by hybridizing the 5′ end of the selector probe to an internal part of the genomic fragment. Thus, sequences located between the restriction site and the target sequence can be efficiently removed.

Selector probes have been applied for analysis of copy-number variation via an assay known as multiplex ligation-dependent genome amplification (MLGA) (70). In MLGA, an assay similar to MLPA, the selected target fragments are designed to differ in length and thus to permit quantification by capillary gel electrophoresis. The relative peak area of the amplification product represents target abundance. By normalizing peaks to reference loci, one can identify differences in gene copy number between a sample and a reference. This assay has been used to locate the boundaries of a duplication by selecting a set of probes distributed across a candidate region for the duplication (71).

6.1.4. In situ genotyping assays. The suitability of padlock probes for in situ analysis was illustrated in the first paper to describe these reagents (16). The padlock probe technique was later used to genotype a single-nucleotide variant in repeated centromeric sequences on human chromosomes 13 and 21 (15), and it was later extended to repeated sequences on chromosomes 7, 12, and 15 (59, 72). These studies relied on padlock probes directly labeled with haptens or

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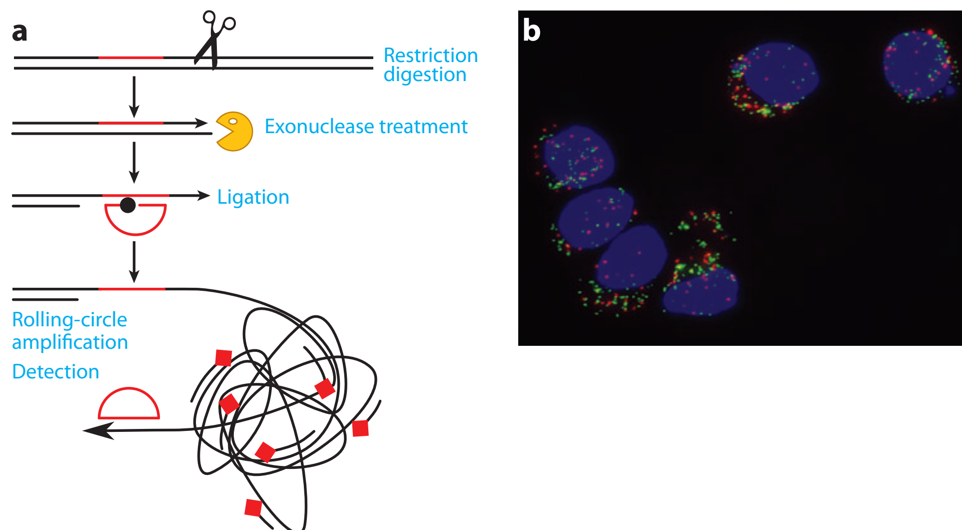

Figure 4

In situ padlock probe analysis for imaging the location of DNA sequence variants in cells and tissues. (*a*) Genomic DNA in cells prepared for microscopy is restriction-digested downstream of the target sequence. The target site is then made accessible by removing the complementary strand using a 5′ exonuclease. Next, a padlock probe is added. The probe is ligated upon hybridization to its target sequence. Finally, the free 3′ end of the target strand primes a rolling-circle amplification (RCA) reaction, and the concatemeric product is visualized via hybridization of fluorescent oligonucleotides. Thus, the amplified molecule forms as a covalently linked extension of the target strand. (*b*) In situ padlock probing for mitochondrial genotyping of two single-nucleotide variants in mitochondrial DNA in the G55 cell line. This cell line is heteroplasmic for the sequence variants and thus has some mitochondria with the normal sequence and some with the mutation. The red signals represent padlock probe–based detection of the normal sequence variant, and green signals represent the mutation.

fluorophores, but the signal was insufficient to detect single-copy sequences due to the background generated from nonspecifically bound probe molecules.

RCA proved capable of amplifying signals from single-copy sequences in situ, as was first demonstrated in cytological nuclear halo preparations, but the signals were reported to be low and variable (48). Target sequences that remain topologically linked to circularized probes greatly inhibit RCA (64). To alleviate this problem, Christian et al. (73) developed an assay in which the target DNA was restriction-digested close to the detection site. The restriction-digestion was cleverly combined with exonuclease digestion to remove the nontarget strand, thereby avoiding the need to denature the target sequences by heat treatment.

The enzymatic target–preparation technique was later modified so that the RCA reaction could be primed by the target strand itself. Thus, the reaction product became a covalently linked extension of the target molecule (**Figure 4*a***) (74). This target-primed RCA resulted in improved detection efficiency and a robust protocol for genotyping point mutations in mitochondrial DNA in both cultured cells and tissue sections (**Figure 4*b***). Through counting of RCPs from differentially labeled padlock probes, this method also proved suitable for relative target quantification. This technique has since been applied on a larger scale in cultured cells through use of image-analysis software (75, 76).

With a detection efficiency of 10%, use of the target-primed RCA approach to detect single-copy genes remains limited. However, the method has been used (*a*) to detect high-copy nuclear

sequences for analysis of double-stranded DNA breaks in cultured cells (77), (*b*) in the so-called comet assay (78), and (*c*) to investigate highly repeated sequences in chromosome spreads (79). The method has also recently been used for in situ detection of the intracellular microorganisms *Anaplasma* spp. in infected cultured cells (80). Padlock probes and RCA have also been used to detect DNA sequences in bacterial cells (81, 82). Most notably, Smolina and colleagues (83, 84) detected bacterial chromosomal DNA by locally opening the target site using peptide nucleic acid openers under nondenaturing conditions.

Compared to DNA strands, RNA molecules are less efficient templates for circularization of padlock probes by DNA ligases (38, 39). To circumvent the need for RNA-templated ligation, a new circularizable molecule, termed the turtle probe, was introduced for the detection of nonpolyadenylated RNA molecules in situ (85). Turtle probes are linear oligonucleotide probes that are ligated into circular molecules, independent of target recognition, but that are templated by an internal probe sequence. This method is so far limited to abundant nonpolyadenylated RNA molecules, and a more general approach is needed for ligase-based detection of mRNA in situ to confer advantages over traditional in situ hybridization methods.

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6.2. Ligase-Based Target Preparation for Parallel DNA Sequencing

Recent years have brought a dramatic development in very high throughput sequencing technology (86). Despite the greatly reduced cost of sequencing, it remains preferable to enrich sequences of interest before proceeding to sequence. Accordingly, there is a need for methods capable of extracting many selected parts of a genome, such as all exons from a large set of genes, to feed into the new, powerful sequencing instruments. Traditionally, this enrichment has been performed on one or a few sequences at a time using PCR, but this method is poorly suited for multiplexing, thus necessitating large numbers of separate reactions.

6.2.1. Selector probes. The selector probes described above for copy-number measurement are also suitable for multiplex target preparation for DNA sequencing. The two ends of the probes, which hybridize to both ends of genomic restriction fragments, provide high specificity while avoiding the risk of cross-reactivity seen in multiplex PCR with many pairs of PCR primers (**Figure 3*b***). Dahl and coworkers (87) showed that this method, coupled with massively parallel pyrosequencing (88), can be applied to amplify all the coding sequence and flanking regions from 10 genes implicated in colorectal cancer.

Fredriksson and coworkers (89) modified the selector method to select fragments generated by a multiplex PCR, rather than the genomic DNA itself. 170 primer pairs were designed to amplify a coding sequence of 10 genes by PCR. However, such a reaction is also expected to generate many undesired amplicons that are not products of cognate primer pairs. By designing so-called gene-collector probes that guide the circularization of products having cognate primers at the ends, one can collect all intended fragments and further amplify them by hRCA, while avoiding amplifying irrelevant amplicons.

6.2.2. Gap-fill padlocks. In another approach, gap-fill padlock probes are used to amplify selected areas of the genome. The ends of the probes are designed to hybridize at a distance, for example, flanking entire exons. A polymerase is used to extend across the region spanned by the ends of the probe, and a ligase then joins the extended sequence with the 5′ end of the probe (90, 91). This results in a set of circularized genomic DNA molecules, all containing a general sequence that can be used for amplification with a standard primer pair. In a study by Porreca et al. (91), 55,000 probes were applied to target a majority of the protein coding sequences in the genome. The probes were

synthesized through enzymatic amplification of a probe library synthesized on a microarray (92). The products were then analyzed by sequencing through use of the Illumina Genome Analyzer, and results showed that about 10,000 of the 55,000 targeted sequences were amplified and sequenced at least once. The poor and uneven representation shows that this technique needs to be further developed, as do other methods for parallel target preparation. In a smaller study, a more even representation was achieved when gap-fill padlock probes were applied for amplification of 485 exons (93). The authors showed that this representation could have resulted from the use of padlock probes that roughly matched the size of the selected fragment, potentially facilitating the gap-fill reaction.

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6.3. Sequencing by Ligation

An approach to obtain DNA sequence information by ligation of short oligonucleotide probes was first suggested by Mirzabekov and colleagues (94). Later, Pritchard & Southern (37) showed that a ligase could be used to extend a primer with several nonamer oligonucleotides along a template in a single ligase-driven DNA polymerization reaction.

The first highly parallel sequencing by ligation technique was performed by Brenner and colleagues (95). In this massively parallel signature sequencing approach, the sequencing templates consist of millions of beads, each harboring a clonally amplified target DNA sequence. These templates are produced in a water-in-oil emulsion PCR that contains beads, where one of the PCR primers is attached to a bead. Signature sequences of 16–20 bp were obtained by repeated cycles of enzymatic cleavage with a type IIs restriction endonuclease, adaptor ligation, and sequence interrogation by fluorescence-labeled hybridization probes. This technique has primarily been used to achieve massive digital quantification of gene expression.

Church and coworkers (96) developed a ligase-based sequencing strategy based on extension of primers with short allele-specific oligonucleotides. As in the previous approach, the sequencing template consists of a library of clonally amplified target molecules attached to beads. The sequence information was acquired in an iterative process through which mixtures of random fluorophore-labeled nonamers were hybridized and ligated to the primer. In the first iteration of the process, the first position after the primer was identified. The color of the ligated nonamers represents the base present in the interrogated position. Before reading the next base, the ligation product was stripped from the beads, and the process was repeated for the next seven positions in the random nonamers.

Another sequencing by ligation approach is used in the SOLiD instrument (sequencing by oligonucleotide ligation detection) developed by Applied Biosystems. Again, in this technique, the template library consists of molecular clones generated through water-in-oil emulsion PCR. The SOLiD method uses a pool of fluorescence-labeled probes to decode the nucleotide sequence on each bead (**Figure 5**): The octamer oligonucleotides consist of six random positions, followed by one of the 16 possible dinucleotide combinations at their 3′ end. Individual oligonucleotides are labeled with one of four fluorophores at the 5′ end, each representing four different dinucleotides. A probe from this pool can only be ligated to the phosphorylated universal sequencing primer if its two 3′ nucleotides hybridize with the template. At this point, the color of the ligated probes at each bead is recorded. The fluorophore is removed by chemically cleaving the ligated probe after the third random nucleotide, forming a new phosphorylated 5′ end. This cycle is repeated five to seven times, generating partial sequence information for 2 out of 5 bp. The ligated strand is stripped and the process repeated with four universal sequencing primers shifted 1–4 nt. Along with a known base located at the beginning of the sequence, a total sequence of a few tens of nucleotides can be decoded for each amplified molecule from the five series of ligation reactions

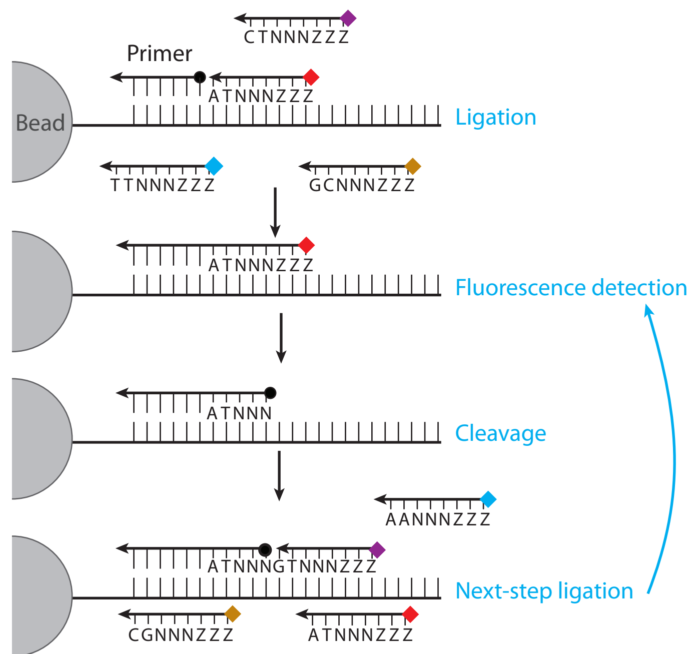

Figure 5

DNA sequencing by ligation. In the SOLiD (sequencing by oligonucleotide ligation detection) procedure, sequencing from each primer identifies one-fifth of the nucleotides along individual target sequences. Thus, complete information can be assembled for each sequence by consecutively using five staggered primers. 3′ ends of DNA strands are indicated by arrowheads, and 5′ phosphates at ligation sites are shown as filled circles. Fluorophores present in four colors are shown as diamonds.

(97). At present, a typical run of the SOLiD instrument generates 240 million short reads with 6 Gb of sequence information, but the sequence production rate increases rapidly, as is the case for other next-generation sequencing techniques.

6.4. Measuring DNA-DNA Interaction

6.4.1. 3C. Ligation reactions are used to reveal the proximity between genomic regions in the nuclei of cells. The chromosome conformation capture (3C) assay was developed to analyze the spatial organization of chromosomes and genomic regions. First, cells are treated with formaldehyde, cross-linking DNA-protein complexes that hold specific genomic regions near one another. The cross-linked chromatin is digested by a restriction enzyme, which creates sticky ends. The digested chromatin fragments are then ligated under very dilute conditions, favoring ligation between DNA fragments cross-linked to one another. Next, the cross-links are reversed, and ligated products are quantified by PCR with primers specific for remote genomic regions that are suspected to interact in the nucleus (**Figure 6**) (98). The amount of PCR product is a measure of specific interactions between two investigated genomic regions in the fixed nuclei.

6.4.2. 4C and 5C. The 3C method has been modified to capture and screen all the DNA fragments that physically interact with a certain DNA fragment. There exist two variants of this method, both known as 4C. The aim of circular chromosome conformation capture (4C) is to ligate both ends of two DNA fragments, which are maintained in contact by cross-linking

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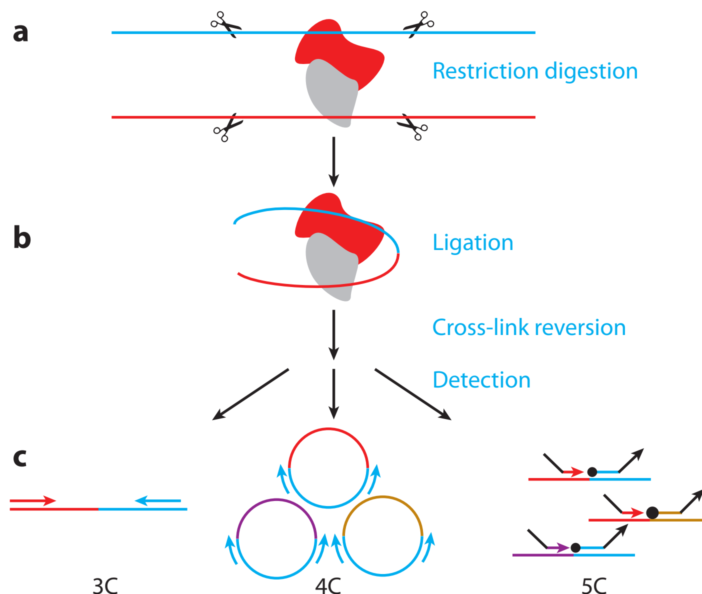

Figure 6

Chromosome conformation capture (3C) technologies for investigating proximity between different genomic sequences in the nucleus. (*a*) Cross-linked genomic DNA is restriction-digested. (*b*) The fragments are ligated to one another, and the cross-links are then removed. Finally, the resulting fragments are analyzed by one of the following methods. (*c*) Polymerase chain reaction (PCR) is performed between known fragments, succeeding if they are joined (*left*). Circular DNA fragments form by ligation of both ends when two separate fragments have been kept in contact by cross-linking. PCR with primers derived from the known fragment (*shown in blue*) amplifies interacting sequences that become ligated (*center*). Any interactions between sets of fragments of interest can be investigated by using specific ligation probes for the ends of their restriction fragments (*right*). All of these ligation products can then be amplified, via the standard primer sequences included in the ligation probes, and recorded. Abbreviations: 4C, circular chromosome conformation capture; 5C, chromosome conformation capture carbon copy.

nuclear protein-DNA complexes, into one circular DNA molecule. After reversing cross-links, any DNA fragments captured in the DNA circles along with a fragment of interest can be amplified using two specific primers extending from the ends of the fragment of interest (**Figure 6*c***). The captured fragments can then be identified by microarray hybridization or sequencing (99). The chromosome conformation capture-on-chip (4C) method differs in that a second restriction enzyme digestion and ligation step are used after reversal of the cross-link (100).

Chromosome conformation capture carbon copy (5C) is a further development that allows the user to study interaction networks among many different genome segments. A 3C library converted to a 5C library, then interrogated by microarray or high-throughput sequencing, allows concurrent analysis of several interaction partners of interest in all combinations (**Figure 6*c***) (101).

6.4.3. chIP-loop assay. The chromatin immunoprecipitation–combined loop (chIP-loop) assay combines chIP with the 3C method to investigate protein-mediated DNA interactions. Here, chromatin is cross-linked and restriction-digested. Fragments that are cross-linked to a protein of interest are immunoprecipitated and ligated. The resulting 3C library can be probed by PCR to investigate whether the proteins have contributed to bringing the regions in proximity with one another (102).

6.5. Protein Analysis via the Proximity Ligation Assay

Proximity ligation assay (PLA): technique for detecting macromolecules via affinity reagents with attached DNA strands, giving rise to reporter DNA strands by DNA ligation

6.5.1. 2PLA and 3PLA. Proximity ligation is a general method for DNA-assisted analysis of target molecules or complexes of molecules for which affinity probes are available, and the assay is of particular value for protein measurements. In the proximity ligation assay (PLA), specific affinity binders such as antibodies or DNA aptamers are conjugated with oligonucleotides having free 3′ or 5′ ends, creating proximity probes. When a pair of proximity probes binds to one protein, the 3′ and 5′ ends of their oligonucleotides are brought into proximity and can then be joined by enzymatic ligation, guided by a connector oligonucleotide that serves to juxtapose the ends of the oligonucleotides on the affinity reagents and to template ligation (2PLA) (**Figure 7*a***). Thus, protein-detection reactions produce surrogate DNA markers that can be amplified, identified, and quantified via techniques for DNA detection, such as real-time PCR and RCA. The requirement for dual

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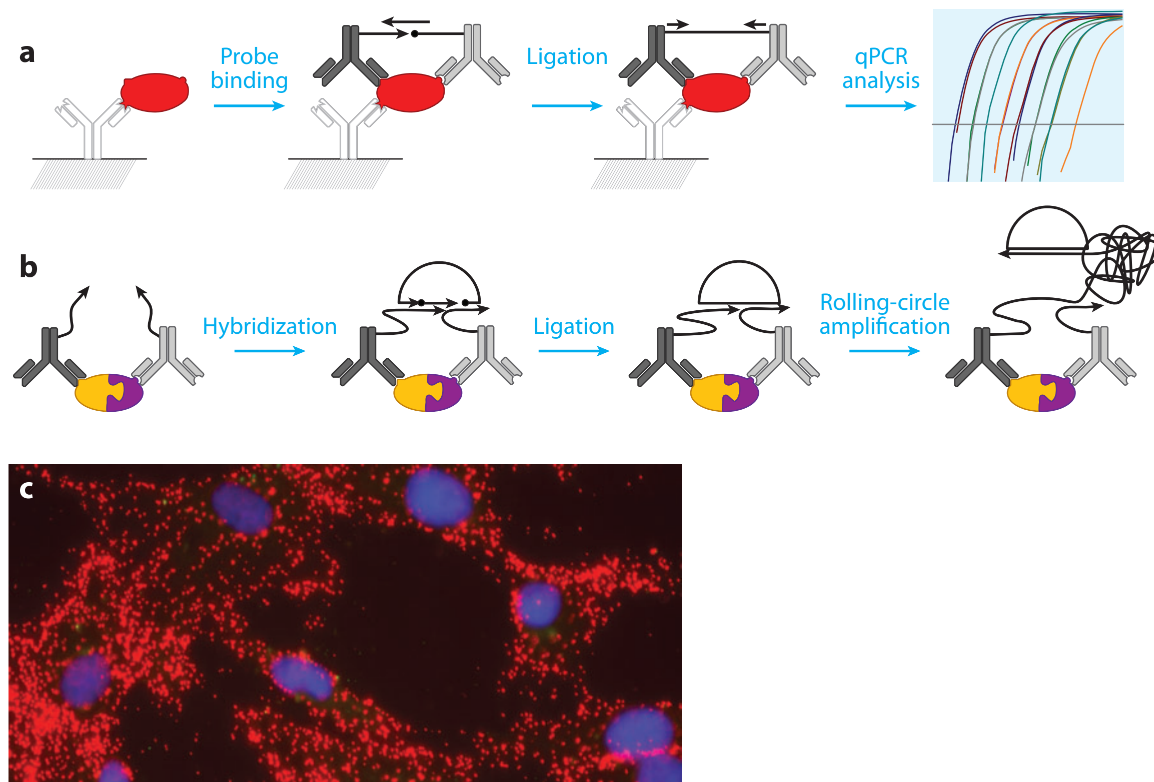

Figure 7

Proximity ligation assays (PLAs) for protein detection in solution and in situ. (*a*) In solid-phase PLA, target protein molecules are first captured from a sample by surface-immobilized antibodies. After a washing step, a pair of antibodies with attached oligonucleotides binds to the antigen, bringing the 5′ and 3′ ends of the oligonucleotides close enough to be ligated to each other and templated by an added oligonucleotide. The amount of ligation product is then assessed by quantitative polymerase chain reaction (qPCR), and the results are interpreted as a measure of the amount of the analyte. PLA can also be performed without solid-phase capture, enabling sensitive detection without the washing steps. (*b*) In situ proximity ligation for localized protein detection. A pair of interacting proteins, each bound by one antibody with attached oligonucleotides, brings these oligonucleotides close enough to each other to act as templates for the ligation of two more oligonucleotides into a DNA circle. One of the antibody-bound oligonucleotides can then act as a primer for rolling-circle amplification. The concatemeric product is visualized by hybridization of fluorescent oligonucleotides. (*c*) Detection of phosphorylated platelet-derived growth factor receptor beta in cultured cells by in situ proximity ligation via one antibody directed against the receptor protein and another antibody specific for a phosphorylated residue in the receptor protein.

recognition by the proximity binders provides high-specificity detection with minimal background, and the subsequent efficient amplification of ligated oligonucleotides ensures high sensitivity.

Proximity probe: an analyte-binding reagent coupled to an oligonucleotide arm

In a homogenous assay where no washes or separations are required, subpicomolar levels of protein targets can be detected in assays that are similar to reverse transcriptase real-time PCR (103, 104). Solid-phase PLA, by contrast, requires washes, but it allows large sample volumes to be interrogated and minimizes matrix problems from difficult samples that may contain nucleases, proteases, or colored products that can compromise fluorescence detection. Protein targets are first captured by affinity binders immobilized on a surface, as in sandwich ELISA (enzyme-linked immunosorbent assay), while other sample components and excess proximity probes are removed by washing steps before ligation and amplification. Thus, solid-phase PLA further improves the sensitivity and specificity of detection of proteins or protein complexes, enabling detection of individual pathogens (105).

In a variant of PLA known as 3PLA, a third proximity probe is included, and these reactions thus require (*a*) binding by three affinity reagents and (*b*) two ligation events to form a PCR template that reports the presence of proteins. 3PLA exhibited a further increase in sensitivity over 2PLA and resulted in a signal greater than background from as few as 60 recombinant vascular endothelial growth factor (VEGF) molecules (106).

PLA is also promising for parallel measurement of large sets of proteins in a sample. Multiplex antibody sandwich reactions are plagued by rapidly increasing cross-reactions between noncognate pairs of antibodies with increasing multiplexing. In PLA, however, it is possible to constrain ligation or amplification reactions so that only the desired reaction products are observed, potentially permitting high levels of multiplexing. Fredriksson et al. (107, 108) established the first multiplexed PLA with a series of sevenplex assays, each measuring proteins in 1-μL aliquots of serum with excellent sensitivity.

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6.5.2. Detection of protein interactions and modifications. Because of its dependence on two or more recognition events, PLA is beneficial for detection of protein interactions and modifications, and determinants several tens of nanometers apart can be shown to be located in proximity. The assays can thereby provide valuable insights into basic biomolecular processes and pathological mechanisms. As an example, the marker of prostate cancer, prostate-specific antigen (PSA), can interact with proteins such as antichymotrypsin and alpha 1–protease inhibitor. 3PLA has been used to measure the interaction of these proteins so as to evaluate the interaction between PSA and one of its inhibitors (106). In analogy to the detection of PSA and its interaction partners, PLA in solution has also been used to monitor binding of VEGF-A to its two receptors, VEGFR-1 and VEGFR-2, and to monitor disruption of their interactions via a number of inhibitors (109). Furthermore, Jarvius et al. (110) have used PLA to investigate posttranslational modifications such as specific receptor phosphorylations by using one binder against a protein and one against its modifications. This method can be extended to a wide range of interacting macromolecules and has been used to investigate target sequence specificity of transcription factors (111).

6.5.3. In situ proximity ligation assay. PLA can provide information about the functional status of proteins, namely the presence of splice variants and posttranslational modifications, and information regarding participation in complexes. PLA can also offer insights into the distribution of the detected molecules in tissues and cells. By combining PLA with RCA, a prominent localized detection signal can be obtained at the site of target-molecule recognition (**Figure 7*b***). For this purpose, fixed cells or tissue sections are first incubated with pairs of proximity probes. If these proximity probes bind sufficiently close to each other, then their attached DNA strands can template ligation reactions that join the ends of two oligonucleotides, which are added in solution and designed so

that they will form a circular molecule via two ligation reactions. Next, these DNA circles are replicated by RCA via one of the antibody-bound oligonucleotides of the proximity probes as a primer. The resulting RCP is thus an elongation of the PLA probes and contains a sequence composed of a series of complements to the circular DNA, anchored at the site of antibody binding. The localized amplification products are finally visualized using fluorescence-labeled oligonucleotides that hybridize to the RCP (**Figure 7*b***) (112). The intensity of the brightly fluorescent spots, representing individual RCPs, reduces problems with background fluorescence, and individual fluorescent signals can be quantified digitally for objective evaluation of the results (**Figure 7*c***).

The requirement for dual recognition by PLA probes greatly improves the selectivity of this method compared to methods based on binding by single affinity reagents, such as immuno-RCA or immunofluorescence, where any specifically or nonspecifically bound detection reagent can give rise to detection signals. The pair of PLA probes can be selected either to bind to the same molecule for increased selectivity of detection or to target two different proteins in a complex, thereby demonstrating their interaction.

In situ PLA has been used to study heterodimerization between (*a*) c-Myc and its binding partner Max (112, 113), (*b*) inositol 1,4,5-triphosphate receptors and protein kinase B/Akt (114), (*c*) CD45 and CD3, CD4, or CD8 (115), and (*d*) aurora B kinase and microtubules (116). Analogous to PLA-based detection of molecules in solution, in situ PLA can be designed to require more than two recognition events for the formation of an amplifiable ligation product, allowing one to visualize multicomponent interactions (for instance, c-Myc and Max transcription factor complexes located adjacent to RNA polymerase II) (112).

In situ PLA has also been used to study posttranslational modifications via one antibody directed against platelet-derived growth factor receptor beta and one antibody binding the modification, in this case a phosphorylated tyrosine residue of the ligand-stimulated receptor (110). In this experiment, secondary proximity probes were used, and the samples were first incubated with a polyclonal rabbit antiserum against the receptor and a mouse monoclonal antibody directed against the phosphorylated residue. Next, two PLA probes directed against rabbit and mouse immunoglobulin, respectively, were added. With this approach, generally applicable secondary PLA probes can be used for in situ PLA as long as the pair of primary antibodies is derived from two different animal species, which eliminates the need for constructing antibody–oligonucleotide conjugates for all primary antibodies.

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6.6. DNA Ligase-Based Readout Formats

6.6.1. Homogeneous amplified single-molecule detection. As described above, it is possible to achieve digital quantification of biomolecules with single-molecule sensitivity by employing RCA of circles that have been generated in situ in a strictly target-dependent manner through analytical ligation reactions. The reactions can also be performed in solution phase, where the single-stranded concatemer products that are generated in RCA spontaneously collapse into submicrometer-sized coils of DNA (117). The local concentration of fluorescence-labeled oligonucleotide probes hybridizing to the repeated RCP can be as much as 1000-fold higher than in the surrounding solution (118), and the RCPs are therefore readily visible through standard fluorescence microscopy (117, 119). Due to the high signal-to-noise ratio, the number of RCPs in a solution can be rapidly determined by pumping the labeled products through a microfluidic channel mounted in a confocal fluorescence microscope operating in line-scanning mode. The data files are digitized in a robust manner because the threshold for identifying RCPs can be set well above the background noise level (120). The number of RCPs correlates very well with the number of target molecules in a solution, as the generation of RCPs strictly depends on the presence of DNA circles that form

in target-dependent probing reactions generated by, for instance, padlock and proximity probes (120). The digital nature of the approach allows high quantitative precision, which has been measured at ∼3%. By equipping the probe sequences with target-specific tags, the RCPs can be labeled with different fluorophores, allowing for multiplexing without affecting the quantitative dynamic range of the readout. Discrete objects are recorded, thus avoiding problems with spectral cross-talk. This approach to detecting fluorescence-labeled RCPs in solution phase has been applied for selective detection of closely related bacterial species, *Vibrio cholerae* and *V. fischeri* (120).

The size of RCPs allows other interesting modes of detection. In one approach, bound RCPs modified the rotation properties of small magnetic beads. The measurements were performed in a superconducting quantum interference device. This method permits excellent quantitation, with a sensitivity in the low-picomolar range. However, with a miniaturized device, the measurement sensitivity would exceed these levels (121, 122).

6.6.2. Dual-tag microarrays. As we discussed above, both padlock and proximity ligation reactions lend themselves to parallel analyses of large sets of target molecules, saving time and sample and providing internally controlled measurements. To preserve assay specificity and dynamic range in multiplex measurements of nucleic acids and proteins, one can use ligase-based reactions when evaluating reaction products on oligonucleotide microarrays in the form of so-called dual-tag microarrays (DTMs). This technique has been demonstrated by detecting nucleic acids using padlock probes or proteins with PLA, then cleaving the amplified ligation products to leave monomer DNA strands with tag sequences at each end. Upon their addition to an oligonucleotide microarray, the monomers were ligated to form DNA circles when hybridizing to the correct immobilized oligonucleotide, and this reagent then served as a primer for an on-chip RCA for enhanced fluorescence detection (123).

This technique was applied for detection of RNA via complementary DNA, resulting in a limit of detection of just 30 complementary DNA molecules—an improvement on the order of 100,000 over the limit of detection of direct microarray hybridization. Furthermore, the DTM approach's linear dynamic range extended to nearly 10^5-fold, and nonspecific background on arrays was largely eliminated due to the stringent requirement for hybridization of two tag sequences and enzymatic ligation.

7. OTHER LIGASE-BASED PROCEDURES AND FUTURE DEVELOPMENTS

The ability to join synthetic DNA strands through use of ligases continues to yield interesting applications in a range of areas. Since the 1970s, genes have been constructed via ligation of synthetic oligonucleotides, and lately whole-bacterial genomes have been prepared via stepwise assembly from short chemically produced strands (124). As discussed herein, the ability to progress from affinity interactions to covalently bonded structures via ligation has also been applied for trapping molecules in microarrays, guided by molecular codes included in DNA molecules (123). This unique form of molecular construction at atomic resolution has also proven useful for nanofabrication of objects that range from cubical DNA molecules to nanoscale smiley faces (125, 126). Finally, probe ligation has even been extended to therapeutic applications as a means of putting covalent DNA locks on plasmids in live bacteria in a sequence-specific manner (127).

With regard to analytical probe ligation reactions, the focus of this review, several trends will likely lead to further developments. It is now possible to extend ligase-based assays to analysis of RNA and even protein molecules. New high-performance assays may herald a broader range of biomarkers that can signal disease, including covalent and noncovalent complexes of interacting

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macromolecules and proteins with specific posttranslational modifications that can only now be readily scored in convenient assays. As discussed herein, the ability of ligase-based assays to yield sequence-coded reaction products invites multiplex applications for monitoring large sets of target molecules. Such assays can be readout via microarrays or by deep-sequencing large numbers of individual tag sequences for precise digital measurements of detected molecules.

Given opportunities for increased specificity, ligase-based assays will push limits of detection toward increased sensitivity. Such opportunities arise from the requirement for multiple recognition events that serves to reduce nonspecific signals. This can enable analyses of individual molecules, which will illuminate temporal and spatial variations in the processes taking place in individual cells (74, 112).

One final area where ligase-based assays hold a great but so far untapped potential is in high-performance assays for point-of-care. The focus of future diagnostics will likely shift from central laboratories toward doctors' offices or even toward the medicine cabinets in people's homes. By suitably miniaturizing and simplifying ligase-based assay procedures, an increasing range of advanced tests could be performed that yield excellent performance with minimal or no special equipment. Researchers have recently taken a step in this direction by integrating some of the required steps into a microchip format (128).

In conclusion, increasing insights into the nuts and bolts of ligase-based molecular reactions permit the construction of new valuable molecular assays. These new techniques will continue to improve our knowledge of biological processes in research and diagnostics.

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SUMMARY POINTS

1. Since the early days of ligation science in the 1960s, ligases have been used to analyze a wide variety of biomolecules, including DNA, RNA, and proteins.
2. Ligase-based assays enable highly specific and sensitive analyses of very complex biological samples. For instance, probe ligation allows detection of SNPs in total genomic DNA.
3. Ligase enzymes from diverse organisms are available to meet the needs for particular assay conditions. They differ in properties such as size of enzyme footprint, substrate specificity, and optimal reaction temperature.
4. PLA permits the use of DNA-amplification techniques for protein analysis in cells, tissues, and solutions.
5. Ligases can act as molecular information integrators, requiring several recognition events for a signal to occur. They can be used in assays for demonstrating phosphorylation of a particular receptor in tissue sections.
6. Ligase assays are suitable for multiplex detection of analytes. More than 10,000 SNPs have been genotyped in parallel from 500 ng of genomic DNA.

FUTURE ISSUES

1. Systems biologists will need ligase-based assays to collect global information.
2. Application of multiplexed assays will be essential for high-throughput analyses of macromolecules in precious biobank samples.
3. It will be crucial to develop high-performance point-of-care assays for diagnostic use.

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DISCLOSURE STATEMENT

U.L. and M.N. are founders and co-owners of Olink AB. M.N. is also a founder and co-owner of Q-linea AB and Olink Genomics AB. Olink holds patents for some methods presented in this article. The other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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