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In situ ligation: A decade and a half of experience

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Abstract

The *in situ* ligation (ISL) methodology detects apoptotic cells by the presence of characteristic DNA double-strand breaks. A labeled double-stranded probe is ligated to the double-strand breaks *in situ* on tissue sections. Like the popular TUNEL assay, ISL detects cells in apoptosis based on the ongoing destruction of DNA by apoptotic nucleases. In comparison to TUNEL, it is more specific for apoptosis versus other causes of DNA damage, both repairable damage and necrosis. In the decade and a half since its introduction, ISL has been used in several hundred publications. Here we review the development of the method, its current status, and its uses and limitations.

Keywords

ISL; ISOL; TUNEL; apoptosis; *in situ* assays; pathophysiology; nucleases

1. Introduction

In the early 1990s, the authors were studying the role of cell cycle inhibitors such as p21WAF1/CIP1 in tissue damage and aging (1). As part of these studies, we needed an accurate measure of the incidence of apoptosis in the tissues we were studying. This is a frequent issue in biomedical science and pathological diagnosis: the need for an accurate measure of the number and location of apoptotic cells in fixed tissue. At the time, and continuing to the present day, the popular method for detecting apoptotic cells was the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) technique (2). This labeling method depends on the ability of terminal deoxynucleotidyl transferase (TdT) to add nucleotides to breaks in DNA. During the late stages of apoptosis, double-strand breaks are produced when activated nucleases cleave DNA. Terminal transferase is used in this assay to add labeled nucleotides to the 3’ ends of cleaved DNA molecules, thus providing a sensitive assay for detecting apoptotic cells in tissues (*see* Note 1).

In our experiments, it soon became apparent that many cells that were TUNEL positive could not in fact be undergoing apoptosis. In particular, in studies of the adrenal cortex, we found that some treatments caused large numbers of cells to become TUNEL$^+$ (1). Despite this, the gland did not disappear over the next few days – as would have been expected if such a large portion of the gland were comprised of cells that were actually in a terminal phase of cell death. Instead, the gland appeared to make a full recovery. This indicated that the strand breaks detected by the TUNEL assay were not indicative of apoptosis, but represented sites of temporary damage and potential repair (*see* Note 2).

[1]Because the substrate for apoptotic nucleases is DNA in chromatin, not naked DNA, specific double-strand break structures are predicted to occur. Specifically, double-strand breaks with single 3’ overhangs are a signature for apoptotic DNA cleavage (4).

We then set out to devise an assay to detect strand breaks in apoptotic cells that would be more specific than the TUNEL assay. We thought of adapting the concept of *in situ* detection of DNA strand breaks by using a process that did not depend on terminal transferase end labeling. The distinctive feature of apoptosis is the presence of double-strand breaks, which may have either blunt or staggered ends. We wanted to label double-strand breaks in such a way that the double-stranded nature of the DNA ends would become an essential part of the labeling process; single-stranded DNA ends would not be labeled. We developed the idea that double-strand breaks could be labeled by ligation of a double-stranded DNA tag. This method is in essence an *in situ* adaptation of ligation methods used commonly in molecular biology, both during subcloning procedures and in analytical procedures, such as ligation-mediated PCR (3) (*see* Note 3).

In our first experiments we used PCR to make digoxigenin-tagged double-stranded fragments that could be ligated to DNA double-strand breaks in apoptotic cells (4). These probes were incubated with deparaffinized or frozen sections of tissue in a mix of buffer, T4 DNA ligase and ATP (required for the ligase reaction). The probes were allowed to become covalently attached to available sites on the section, and unattached probes were then washed away. The attached probes were detected by an antibody against digoxigenin or were directly observed by fluorescence microscopy. Practical tests of the method showed that it did indeed label apoptotic cells specifically. As a positive control we used rat thymus 24 hours after administration of glucocorticoid, a model for apoptosis well established by previous investigators (5). These experiments resulted in the introduction of a new assay for *in situ* detection of apoptosis.

2. An assay with many names

The *in situ* ligation assay was designed to mark apoptotic cells via detection of two specific types of DNA damage. It selectively labels 5’ phosphorylated double-strand DNA breaks, which have either blunt ends or 3’ single base overhangs. Its basic components are the enzyme T4 DNA ligase and a DNA-based probe, which is ligated to the ends of cellular DNA breaks.

In the initial 1996 paper, where we described the technique using PCR fragments as probes, we did not give the assay a special name (4). This later resulted in an unusual consequence of the ligation assay having multiple different names.

Soon after the assay introduction, as we gained more experience with *in situ* ligation, we realized that the major practical problems were to make sufficient amounts of the PCR product and to purify it away from unincorporated labeled dNTPs, which could produce increased background on the section. We thought that making a probe chemically rather than enzymatically might solve these problems. To that end, we designed a double-stranded oligonucleotide that could be used to label double-strand breaks. The first generation of these hairpin probes had a stem-loop configuration resembling a tennis racket and became known as “looped hairpins” (6) (*see* Note 4). The design offered the advantages of structural

[2]At the time of these experiments there were already many indications that the TUNEL assay was not very specific for apoptosis. Other studies had suggested that necrotic cells stain positively in the TUNEL assay (33, 35).

[3]As a historical note, we developed the ISL method not by using the ligation-mediated PCR example, but instead based on a more general consideration of adapting common molecular biology techniques to *in situ* detection. However, independently a PCR-based method was developed for biochemical detection of double-strand breaks in apoptotic cell DNA (60).

[4]In the first type of oligonucleotide that we designed, we retained a single A 3’ overhang as found in Taq polymerase-derived PCR products. The loop contained 5 deoxyuridine derivatives labeled with biotin. We also used a blunt-ended probe. Although the probe with a 3’ overhang is probably more specific and was used most, its signal is also weaker as compared with the blunt-ended hairpin. In sum, both types of probes can detect apoptotic cells (4, 6, 39); the choice of which to use is best determined empirically in the biological system under investigation.

uniformity and stability. The probes carried five biotins located in the loop area and were easy to prepare in large quantities. However, the loop area had some tendency to stick to sections and the *in situ* labeling procedure still required lengthy washing steps to remove the unligated probe. To address these issues, we designed a new oligonucleotide probe which became known as a "loopless hairpin" (7). In the new probe design, the reactive single-stranded loop was eliminated. In order to avoid steric hindrance problems and to create better conditions for the reaction between biotin and streptavidin in probe detection, the number of biotins was reduced from five to one. The design substantially reduced the cost of the probe and simplified the assay, transforming it into the *in situ* oligonucleotide ligation technique (ISOL), a convenient and robust modification of ISL methodology detecting apoptosis and DNA damage in tissue sections.

These developments came one after the other in short intervals, so all of the different probe designs were put into use in rapid succession and were employed concurrently. As a result the ISL assay is known under many names, depending on the probe design used by a particular research group. In general, these names follow the evolution of the ligation probes. So that when PCR probes are employed, the assay is presented as "*Taq* and *Pfu* labeling techniques" (8–10) or "*Taq* and *Pfu* polymerase *in situ* ligation assay" (11–16). The "looped" probes usage is acknowledged by such names as "hairpin probe assay" (17) or "HPP staining" (18–20). The arrival of the "'loopless" probe and the popular commercial kit (ApopTag® Peroxidase *In Situ* Oligo Ligation kit from Millipore) which used it, resulted in the names of "*in situ* hairpin-1 ligation assay" (21) and "*in situ* oligonucleotide ligation" (ISOL) (22, 23). In addition to those, several more general or exotic names are simultaneously used, such as "*in situ* DNA ligase method" (24), "3'-overhang ligation" (25), "PCR *in situ* ligation assay" (26) and even "the Didenko and Hornsby ligation technique" (25).

We, as many others, prefer the most general title of "*in situ* ligation" (ISL) which is not influenced by variations in probe design (27–31).

3. Technological evolution of *in situ* ligation assay

The *in situ* ligation technique underwent several cycles of re-development since its inception in 1996. The majority of improvements concentrated in three areas: new ligation probes, expansion of detection targets, and increased sensitivity of detection.

3.1. Ligation probes

We have already discussed the three different designs of *in situ* ligation probes. Although with each new design our intent was to develop a probe which would surpass the earlier construct on all counts, yet all of these probe configurations appeared to have their own advantages and limitations. This probably explains why all of them are simultaneously employed by different groups.

The PCR-derived probes contain dozens of tags, which results in high sensitivity because every detected DNA break is labeled by multiple dyes. Another attractive feature of these probes is that they can be easily produced ad hoc when needed in any molecular biology laboratory. Their disadvantage is in the longer and more numerous washing steps because they tend to stick to tissue sections.

The advantage of the loopless hairpin probes, such as those used in the ApopTag ISOL kits, is in the complete absence of nonspecific background staining, as all unligated probes are easily washed away from the sections. Oligonucleotide probes also provide the opportunity to design any kind of double-stranded DNA ends, both for apoptotic cell labeling and for

labeling any types of double-strand breaks in other biological materials. The hairpin configuration offers an efficient solution to the problem of forming double-stranded probes that are uncontaminated by single-stranded DNA. However loopless hairpins only place a single tag at the end of each detected DNA break and, as a result, have the lowest sensitivity. This can be partially improved by using the post-labeling enzymatic amplification of signal (28).

The “looped” hairpins occupy a position intermediate between the PCR-derived and the “loopless” probes. They contain at least five tags but also possess a single-stranded region, which necessitates more vigorous washing of sections.

While the advances in ligation probes focused mainly on background reduction and more specific detection, the other direction in ISL evolution concentrated on increasing the variety of detected types of DNA damage and on co-labeling of other cellular markers.

3.2. Expansion of targets for detection

Per se the ISL assay exclusively detects only two DNA targets: it labels 5’ phosphorylated double-strand DNA breaks which have either blunt ends or 3’ single base overhangs. These particular types of breaks are important because they mark apoptotic cell death and can be used in its discrimination from necrosis. However the spectrum of detectable biological targets and the utility of the assay can be expanded by combining ISL with other assays in dual- and triple-staining procedures. This advantage was explored in recent years by combining the ISL staining with other techniques, such as antibody immunohistochemistry, TUNEL, Klenow polymerase-based labeling, vaccinia topoisomerase-based labeling and T4 DNA kinase section pre-treatment. Such multi-labeling procedures generate more information as compared with single-staining. They increase the variety of detected DNA breaks, show their relative distribution, and co-visualize protein-based markers of cellular processes. The approach is also useful for validating results of individual techniques employed in experiments.

3.2.1. Co-labeling with TUNEL—ISL-TUNEL co-labeling was, perhaps, the earliest used combination. In terms of DNA breaks detection, the popular TUNEL assay labels both single- and double-strand breaks (32). The only requirement for labeling to work is the presence of free 3’OH groups. This assay is frequently employed to label apoptotic cells; however it suffers from low specificity due to the presence of DNA breaks with 3’OH in many other processes (4, 33–37). The combination of TUNEL and ISL permits improved detection of apoptotic cells and better analysis of DNA damage present *in situ*. This co-staining could not be used with PCR-derived probes because 3’OH groups at the unligated ends of the probes would be labeled by TUNEL. In addition, nicks and single-stranded breaks with 3’OH could be present in the long PCR fragments used as probes, thus creating additional priming sites for TUNEL. However, the problem of ISL-TUNEL co-labeling was resolved with the introduction of short hairpin oligo probes, permitting simultaneous visualization of single-strand and double-strand DNA breaks at the subcellular level (6, 28, 38).

3.2.2. Co-labeling with Klenow polymerase—Although on its own *in situ* ligation visualizes only double-strand DNA breaks that are either blunt-ended or possess 3’ overhangs, when modified, it also permits the selective detection of both 3’→5’ and 5’→3’ exonuclease activities *in situ*, via labeling of 3’ and 5’ DNA overhangs of all lengths. We developed this expanded approach for the purpose of comparative analysis of DNA damage in apoptosis and necrosis (39). The technique relies on Klenow enzyme pretreatment of cells before *in situ* ligation. If such pretreatment is performed in the in the presence of dNTPs it

fills up all 3’ overhangs, converting them into blunt ends. However when the pretreatment is done without added dNTPs, it reduces all 5’ overhangs to blunt ends, which can be subsequently detected by blunt end *in situ* ligation. Co-labeling with Klenow polymerase has expanded the utility of ISL, permitting the detailed study of DNA damage *in situ*. Later this method was applied for analysis of free radical-induced DNA damage (29).

3.2.3. Dual detection with T4 polynucleotide kinase pretreatment—Based on the distribution of hydroxyl and phosphate groups at the ends of DNA breaks, two types of DNA breaks can potentially be present in apoptotic cells: 3’OH/5’PO_4 breaks, generated by DNase I and DNase I-like nucleases; and 3’PO_4/5’OH DNA breaks with inverted distribution of these chemical groups, produced by DNase II and DNase II-like nucleases (40–42).

TUNEL and *in situ* ligation can visualize only 3’OH/5’PO_4 breaks. These techniques cannot detect DNA fragmentation produced by nucleases generating 3’PO_4/5’OH breaks, because the TdT enzyme used in TUNEL does not react with 3’ PO_4 ends and T4 DNA ligase in the ligation assay is unable to attach the probe to 5’OH ends in DNA (43, 44).

Therefore several protocols were developed in our laboratory for selective *in situ* labeling of double-strand DNA breaks with terminal 5’OH groups. The earliest of these procedures was a modification of the standard *in situ* ligation approach using a T4 kinase-based conversion of the breaks (45). The assay permitted fluorescent detection of 5’ hydroxyl-bearing double-strand breaks with blunt ends or short 1–2 base long 3’ overhangs.

The technique is based on the conversion of 5’ hydroxyls into 5’ phosphates with the help of the enzyme T4 polynucleotide kinase and their subsequent detection by *in situ* ligation. The procedure is performed in three stages. In the first stage, the unlabeled hairpin oligonucleotides are ligated to the section, blocking available 5’ phosphates, which may be present on the ends of DNA as a result of DNase I type nuclease activity. In the second stage, phosphate groups are added to the 5’ OH ends by T4 polynucleotide kinase. In the third stage, an *in situ* ligation reaction is performed again using the hairpin probes.

The assay can be modified to simultaneously visualize both 5’ phosphates and 5’ hydroxyls using two different fluorophores. In this case, biotinylated hairpin probes (instead of unlabeled hairpins) are used in the first and third stages of the labeling reaction and visualized using different fluorophores. This double-detection assay (45), although highly useful, is time-consuming and we later substituted it with a much faster technology which uses vaccinia topoisomerase I (TOPO) (46, 47).

3.2.4. Dual-detection with vaccinia topoisomerase I assay—The vaccinia topoisomerase-based technique is a close relative of *in situ* ligation, and can be combined with ISL in a dual-detection procedure. First introduced in 2004 (46), combined staining using TOPO and ISL permits co-detection of DNA breaks with 5’ OH and 5’ phosphate groups. In this regard it is similar to the T4 kinase approach discussed above. However the TOPO-ISL labeling is much faster and simpler. Its convenience resulted in a new dual-detection kit from Millipore (ApopTag® ISOL Dual Fluorescence Kit), combining these two techniques.

3.2.5. Co-detection with immunohistochemistry—The possibility of combining ISL with immunohistochemical detection was demonstrated in the first paper introducing the ligase technology (4). The paper showed that no false signal was generated in ISL even after prolonged heating of tissue sections during antigen retrieval, which is often needed for immunohistochemistry. The result was advantageous as compared to TUNEL, which

produces intense artifactual staining in sections heated for antigen retrieval. Soon the dual detection approach combining ISL with antibody immunohistochemistry was tested by others and found useful (48). The co-detection of DNA-based apoptotic markers and various protein-based targets has been reported in several publications (21, 49–51).

4. Advantages and limitations of the ISL methodology

It is clear that the ISL reaction gives researchers in apoptosis a valuable weapon in their arsenal to study this important cellular process. Probably the first contribution of this technology was the clear realization of the imprecise and non-specific nature of the widely-used TUNEL technique and other polymerase-based approaches, which in some situations provided overestimated numbers of apoptotic cells because they labeled other processes. This reinforced the necessity to use morphological and immunohistochemical verification of apoptosis when labeling it via detection of DNA breaks. Understandably, it resulted in a need to use the same strict verification standards in case of ISL too. Several studies addressed this issue and compared ISL with other apoptosis assays for specificity of apoptosis detection and its discrimination from necrosis.

Possibly, the first study which specifically evaluated ISL appeared in 1998 (48). It used ISL with PCR-derived probes, TUNEL and immunohistochemical co-staining. The study concluded that *in situ* DNA ligation was superior to TUNEL and, in association with immunohistochemistry, could readily distinguish apoptosis from necrosis, making it an attractive technique for discriminating these processes.

Since then, various ISL modifications and all three types of ligation probes were tested in many studies. For example, apoptosis detection by using ISL (PCR-derived probe), TUNEL, antibody-based labeling of single-stranded DNA and PARP-1 were compared (52). PARP-1 detection was chosen because this enzyme is a well-known target of the caspase protease activity associated with apoptosis. The study concluded that ISL and antibody-based single-stranded DNA detection, but not TUNEL, closely correlated with PARP-1 expression. Moreover, ISL and single-stranded DNA labeling were significantly more specific for apoptosis than TUNEL. The paper noted that, in contrast to T4 DNA ligase, the TUNEL assay stained cells marked by antibodies against proliferation marker Ki-67 or the splicing factor (SC-35), indicating false positivity. ISL did not stain these same cells (52). This confirmed the earlier work which first noted that cell proliferation, RNA synthesis and splicing interfere with apoptosis detection by TUNEL (53).

In another work, which used the model of neonatal rat hypoxia-ischemia, the spatial and temporal activation of caspase-3 was correlated with three different markers of DNA damage (ISL, TUNEL and monoclonal antibody against single-stranded DNA) and with the loss of a neuronal marker, microtubule-associated protein 2 (54). The study demonstrated that ISL staining with “looped” 3’ dA overhang hairpin probe (HPP) produced the best correlation with apoptotic caspase-3 activation. The same group later expanded their data by employing a slightly different set of approaches for apoptosis and DNA damage detection: Hoechst dye staining, TUNEL and ISL with “looped” hairpin probes. In series of dual- and triple-staining experiments these techniques were combined with co-detection of apoptosis-inducing factor (AIF), which triggers apoptosis in a caspase-independent manner (18).

Yet other research group compared TUNEL, ISEL (*in situ* end-labeling by Klenow polymerase), and ISL using “loopless” hairpins (ISOL). This group studied apoptosis of cardiomyocytes in explanted and transplanted hearts. It concluded that, in contrast to ISL, both TUNEL and ISEL had low specificity in this model. This drawback led to a high prevalence of false-positive results in myocardial studies and was exacerbated by the extreme care required in tissue processing for both these methods (22). While the study

confirmed that the ISL assay detected apoptotic cells in a positive control specimen (involuting rat mammary gland), they found that the number of true apoptotic nuclei in all the hearts under study was extremely low. Indeed this is to be expected, based on the fact that apoptosis in mature organs is typically very low and also that the phase of apoptosis when DNA is actively undergoing cleavage by nucleases is very brief. Thus one always should have a high degree of skepticism in accepting frequencies of apoptotic cells under normal and pathological conditions.

It is gratifying to see that many other groups, unaffiliated with the authors, have independently concluded that ISL is a more specific and sensitive approach for apoptosis detection, surpassing a variety of other *in situ* techniques. Although the pros and cons of these different assays for apoptosis detection are now well-known (27, 28, 33–36, 55), nevertheless it would be true to say that ISL has not replaced TUNEL and other assays.

Quite the opposite, an assortment of multiple techniques is in current use for detecting apoptotic cells. Often this is done by combining several approaches in co-staining. Such multi-assay co-detection compensates for the limitations of individual techniques. The most popular combinations include ISL performed in complex with TUNEL, active caspase-3 detection or other apoptosis-related antibodies, and electron microscopy. These multi-angle assessments have now become standard in delineating contributions of apoptosis and necrosis in various systems.

For the sake of example, we can mention just some of the multi-technique approaches found in the literature. The combinations included ISL, TUNEL, nuclear morphology and immunostaining for p53 and p21 (25); or ISL, TUNEL, H&E staining, and determination of DNA laddering by electrophoresis of labeled DNA (56); or the multitude of other groupings of techniques, all containing ISL (18, 26, 38, 39, 52, 54, 57).

In recent years a new generation of methods aiming to detect apoptosis *in vivo* was introduced and tested (*see* other chapters in this volume). In an interesting application of the technique, ISL labeling was used as part of dual *in situ-in vivo* labeling of apoptosis. It was employed for verification of *in vivo* detection by [^{123}I]Annexin V. The degree of thymic apoptosis was co-determined in the same animals at 6 and 11 h after a single administration of dexamethasone by using two techniques: *in vivo* detection by radioactive Annexin V and *in situ* labeling by ISOL (58). I*n situ* ligation demonstrated a 62- and 90-fold increase of the apoptotic index in thymic cortex at 6 and 11 h. Instead, the Annexin V-based signal fluctuated. It significantly decreased at 6 h and increased only 1.4-fold by the 11 h time-point. The study concluded that the specificity of the apoptotic signal provided by isotopic methods *in vivo* would always require confirmation by complementary *in vitro* techniques that verify the assessment of ongoing apoptosis accurately.

Are there limitations to the assay and reasons why it has not been used more often? In common with the TUNEL assay, ISL depends on the detection of DNA strand breaks. Like the TUNEL assay, it cannot be used as a sole method for apoptosis measurement. It is necessary to have other biological information to indicate that apoptosis is expected in the biological specimens being examined. Unfortunately, too often the TUNEL assay is used as proof of apoptosis, whether or not it is anticipated for other reasons. However, when apoptosis is well established to occur in the tissue under the experimental circumstances being used, either the TUNEL or ISL assays will give equivalent results. For this reason, it is easy to understand why the familiar TUNEL assay has maintained its popularity.

Unlike the TUNEL assay, ISL is more specific for apoptosis versus other causes of DNA strand breaks, such as repairable DNA damage or necrosis. Thus its particular usefulness is under experimental conditions where both apoptosis and other causes of DNA strand breaks

are either simultaneously present or else both likely to occur. For example, in many pathophysiological circumstances of damage to organs, both apoptosis and other damage may be expected. The judicious use of the ISL methodology, in conjunction with other biochemical or cell biological assays, will enable the investigator to reliably determine the true extent of apoptosis present.

Interestingly, we noticed some “favorite” scientific fields where the popularity of ISL is much higher than in others. This refers to the fields of molecular cardiology and neuroscience. ISL was particularly attractive for these fields because they deal with terminally differentiated and highly specialized cells. The ligation technique is well-suited for apoptosis detection in heart and brain cells. In these organs, detection of apoptosis is most challenging and the existing methods, such as TUNEL, are often insufficient and do not provide clear answers about its extent or even existence.

The limitations of the assay stem from the fact that, strictly speaking, ISL is not an apoptosis or necrosis detection technology, but a technique labeling a specific subset of DNA breaks. On the strong side, the validity of these specific DNA breaks as apoptosis markers is well-established. In fact this type of DNA damage is considered more characteristic for apoptosis than any other DNA-based marker. However, as with any other biological marker, it is far from being absolute and always requires cautious interpretation.

5. Current status and perspectives of the ISL methodology

In the decade and a half since the development of the original PCR-based assay, and its subsequent derivatives based on use of modified oligonucleotides, the assay has been used in a large number of publications. It is difficult to estimate the total number with great accuracy, because many articles have used the commercially available ISOL kits (now sold by Millipore Corporation) without citation of the original publications, precluding citation analysis as an accurate way of determining the popularity of the assay. However, based on search engine results, we can determine that the assay has been used in at least several hundred publications. The assay has been adopted by many groups as an alternative to the still hugely popular TUNEL assay. The reasons appear to be those that initially stimulated us to devise the ISL assay: the specificity of the assay for apoptosis versus various other forms of DNA damage.

However ISL is more than just a sensitive assay for apoptosis detection. The distinctive feature of this assay is its unique selectivity for a single molecular target. Unlike other enzymatic *in situ* approaches, such as TUNEL or ISEL, ISL specifically detects only a single type of DNA breaks. Depending on the ligated probe end, it detects only blunt-ended or 3’ staggered DNA breaks with terminal phosphates. Moreover, as we demonstrated, the ISL’s range of detection targets can be expanded to include other characteristic types of DNA breaks. This makes the ligase-based approach a valuable tool for studying a variety of cellular events beyond apoptosis and necrosis. These future new fields of application could include studies in DNA recombination, reparable DNA damage, free radical biology, cell growth and aging.

A necessary pre-requisite for such a technique expansion is the increased sensitivity of DNA breaks detection. This is because much lower numbers of DNA strand breaks, up to a single break per cell, are expected in viable, non apoptotic cells which undergo other processes, such as V(D)J recombination or low level irradiation (59). However, if needed, the intensity of the probe signal can be increased to levels approaching the detection of individual DNA breaks. This can be accomplished by using enzymatic amplification post-ligation, as we discussed (28). This will ultimately permit detection of solitary or very rare DNA breaks.

The practical work in this direction has already started and, we hope, will bring about important new insights.

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