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MINI REVIEW

Use of multicolor fluorescence in situ hybridization to detect deletions in clinical tissue sections

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Abstract

A variety of laboratory methods are available for the detection of deletions of tumor suppressor genes and losses of their proteins. The clinical utility of fluorescence in situ hybridization (FISH) for the identification of deletions of tumor suppressor genes has previously been limited by difficulties in the interpretation of FISH signal patterns. The first deletion FISH assays using formalin-fixed paraffin-embedded tissue sections had to deal with a significant background level of signal losses affecting nuclei that are truncated by the cutting process of slide preparation. Recently, more efficient probe designs, incorporating probes adjacent to the tumor suppressor gene of interest, have increased the accuracy of FISH deletion assays so that true chromosomal deletions can be readily distinguished from the false signal losses caused by sectioning artifacts. This mini-review discusses the importance of recurrent tumor suppressor gene deletions in human cancer and reviews the common FISH methods being used to detect the genomic losses encountered in clinical specimens. The use of new probe designs to recognize truncation artifacts is illustrated with a four-color *PTEN* FISH set optimized for prostate cancer tissue sections. Data are presented to show that when section thickness is reduced, the frequency of signal truncation losses is increased. We also provide some general guidelines that will help pathologists and cytogeneticists run routine deletion FISH assays and recognize sectioning artifacts. Finally, we summarize how recently developed sequence-based approaches are being used to identify recurrent deletions using small DNA samples from tumors.

Introduction

Genomic deletions are one of the major types of somatic mutations that inactivate tumor suppressor genes in human cancers. Some of these inactivation events occur as point mutations or deletions of a few base pairs of DNA that are only detectable by sequence-based methods. However, many of the clinically most important deletions occur as interstitial losses of the entire genomic region containing the tumor suppressor gene. Although there are a number of ways of inactivating a tumor suppressor gene, the end result is nearly always a mutational disruption or loss of coding information, leading to the absence of a functional protein. If protein expression is lost it means that often antibody

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based methods, such as immunohistochemistry may be used to evaluate loss of tumor suppressor genes.

Historically, classical cytogenetic analysis of tumor karyotypes was used to identify interstitial deletions of a chromosomal region known to contain a suppressor gene. However, fluorescence in situ hybridization (FISH) has a greatly improved sensitivity, and the technique is particularly useful when examining pathology tumor tissue preparations in which a suppressor gene deletion is of clinical importance. One of the main advantages of FISH lies in its ability to detect deletions directly in formalin-fixed paraffin-embedded (FFPE) tumor sections, so that gene loss may be examined in the context tumor tissue morphology.

Deletion testing by FISH of solid tumors is usually performed using FFPE tissue sections of a biopsy, with an adjacent section hematoxylin–eosin stained section available for reference purposes during analysis. Enumerating the gene copy number by FISH may be compromised by the partial sectioning or 'signal truncation' of tumor nuclei during the cutting process of slide preparation [1, 2]. The loss of some signals due to sectioning truncations mean that a high background of 'false deletions' can make interpretation more complicated (discussed in detail below). One of the most important sample types to study by FISH or immunohistochemistry in many newly diagnosed cancers are taken by fine needle and core biopsies [3]. Often these initial analyses obtained from needle cores can be used for FISH of immunohistochemistry to provide additional prognostic or diagnostic information depending on the tumor type and the assay of interest. These tiny cylindrical cores of tissue are usually embedded in paraffin blocks. Sections are then cut from the blocks using a microtome typically set to produce sections ranging from 3 to 5 µm in thickness in most laboratories. Sections of 3 µm thickness are particularly challenging for deletion FISH analyses because of loss of parts of the nuclear material during slide sectioning. This issue may have limited the uptake of FISH deletion assays in clinical laboratories however, there are new probe designs that can be used to recognize when slide sectioning losses may be present. In this mini-review, we will summarize the various laboratory approaches taken to determine whether genomic deletion of a tumor suppressor gene has taken place. We will summarize the common FISH methods used to detect the genomic losses encountered in clinical specimens, and we will also present one of the multicolor FISH probe designs that have been used recently to increase the accuracy of FISH deletion assays, even when using thin 3 µm sections. Finally, we will provide some guidelines that may be helpful for laboratories that are considering running FISH deletion analysis of tumor suppressor genes using FFPE tissue sections.

Table 1 FISH deletion assays commonly used in clinical laboratories

Tumor supressor gene or cytoband deleted	Tumour type	Clinical Applications of FISH	Other assays used to determine gene loss
p16/CDKN2A (9p21)	Malignant mesotheliomas	Identification of homozygous losses in cytology preparations helpful in diagnosis.	Loss of protein p16 protein expression by immunohistochemistry.
Co-deletion of chromosome arms 1p36 and 19q13	Oligo-dendriogliomas	Detection of hemizygous losses helpful for diagnosis and associated with favorable outcome.	Deletions are also detected by loss of heterozygosity, quantitative PCR, Multiplex Ligation-dependent Probe Amplification (MLPA), and microarray assays.
TP53 (17p13)	Chronic lymphocytic leukemia (CLL) and multiple myeloma (MM)	Detection of 17p losses using the *TP53* probe associated with poor response to chemotherapy and adverse outcome.	CLL: (i) targeted molecular approaches, such as MLPA; (ii) Specific next-generation sequence gene panels; (iii) Genomic copy number by microarray. MM: Clonality should be considered, many labs still use *TP53* FISH. However, copy number by genomic microarray is often used, particularly in European labs.
PTEN (10q23)	Prostate cancer	Homozygous loss associated with poor outcome. Hemizygous loss unfavorable.	Loss of PTEN protein by immunohistochemistry associated with unfavorable outcome.

Clinically significant gene losses in human cancer

Over the years, loss of heterozygosity analysis, copy-number array comparative genomic hybridization and more recently, whole genome sequencing of tumor DNA, have uncovered a large number of gene deletions, complex insertions and sequence deletions (indels). However, the number of tumor suppressor gene deletion assays being routinely performed by FISH methods using clinical samples is still surprisingly limited. Reduced uptake of these tests for clinical uses may be partly because the diagnostic or prognostic value of loss of a tumor suppressor gene is only just emerging for some tumor types. Also, for many tumors, the best laboratory approach to determining whether a suppressor gene of interest has been deleted has not been established at the rigorous level required for a clinical grade test. Some of the clinically most relevant tumor suppressor gene deletion assays are shown in Table 1. This list is not intended to be comprehensive; we have highlighted assays that illustrate their clinical importance and diversity of the laboratory approaches that are currently being used to detect gene loss.

Homozygous deletion of 9p21, the cytoband harboring the p16/*CDKN2A* gene, has been reported as the most common genetic alteration in malignant mesotheliomas [4]. FISH using probes from the p16/*CDKN2A* gene have been helpful for distinguishing between benign and malignant mesothelial proliferations [5]. More recently the use of immunohistochemistry in combination with FISH has also been helpful in mesothelioma diagnosis [6].

Determination of chromosome 1p36 and 19q13 status by FISH is a crucial step in the diagnosis and the management of oligodendroglial brain tumors (reviewed in ref. 7). Based on current WHO criteria, demonstrating 1p36/19q13 co-deletion is a requirement for confirming the diagnosis of oligodendroglioma [8]. While there are alternative molecular method methods that have been used to identify co-deletion of 1p and 19q, such as loss of heterozygosity or quantitative PCR analysis, the use of deletion FISH seems to be the favored means of testing at the present time [9]. In typical FISH assays probes of different colors are used to label DNA probes to detect 1p36 and 19q13 losses within the interphase nuclei of individual glioma cells from FFPE tissue sections. Changes in the 1p36 and 19q13 probe signal pattern are then compared with controls to determine if the 1p36 and 19q13 co-deletion is present. Co-deletion is strongly correlated with a better response to standard treatment with radiotherapy and chemotherapy as well as a better overall survival.

In chronic lymphocytic leukemia deletions of the 17p13 chromosomal region, which includes the *TP53* gene encoding the p53 protein, is a powerful predictor of resistance to chemotherapy and an overall poor outcome [10]. Abnormalities of p53 are also a common finding with potential therapeutic targeting in multiple myelomas [11]. The presence of hemizygous *TP53* loss and nuclear p53 protein expression by immunohistochemistry in multiple myeloma can be an indication of an adverse outcome [12].

There is a strong correlation between loss of the *PTEN* gene and its protein and adverse pathologic features in prostate cancer (reviewed in [13]). *PTEN* gene loss as determined by both FISH and immunohistochemistry has been associated with biochemical recurrence [14], development of castrate resistant disease [15, 16], and prostate cancer-specific death [17]. Based on recent studies using diagnostic needle biopsies, it has been proposed that an initial analysis of PTEN expression could be carried out using immunohistochemistry. Thereafter, regions of tumor or suspicious areas in the biopsy that have reduced expression of PTEN protein, or are otherwise indeterminate by immunohistochemistry, could then be analyzed by *PTEN* FISH as a reflex test [18]. This general approach would be in keeping with the emerging consensus for several types of oncologic pathology tests such as those for breast [19], lung [6, 20], and melanomas [21], in which the primary immunohistochemistry analysis can be followed by reflex FISH for cases that are inconclusive.

FISH deletion testing using solid tumor tissue sections

In typical FISH deletion assays of solid tumors, the tissue biopsy has been fixed in formalin and then embedded in wax in a mold to produce tissue blocks that are then cut on a microtome (Supplementary Fig. 1A). Some of the earliest probe designs used for tumor suppressor gene deletion assays incorporated two-color FISH probes from two regions on the same chromosome: one specific probe targeting the tumor suppressor gene of interest, and a second control probe, often a centromere probe, that is used to count the number of chromosomes present [22]. Unfortunately, the interpretation of the FISH signals using this basic assay design for FFPE sections can be complicated by signal truncation losses generated by the histological slide making process itself (Supplementary Fig. 1B). Signal truncations are more evident in sections when the genomic distance between the control centromere probe and the tumor suppressor gene probe is large (>3–4 Mb). The significant background percentage of nuclei with 'false deletions' caused by the truncated nuclei in each section requires careful assay design and interpretation since the background truncation losses increase the probability of incorrectly classifying a tumor sample as deleted [2]. It is therefore essential to have matched control tissue and rigorous 'cutoff' values that take these truncation losses into account [22–24].

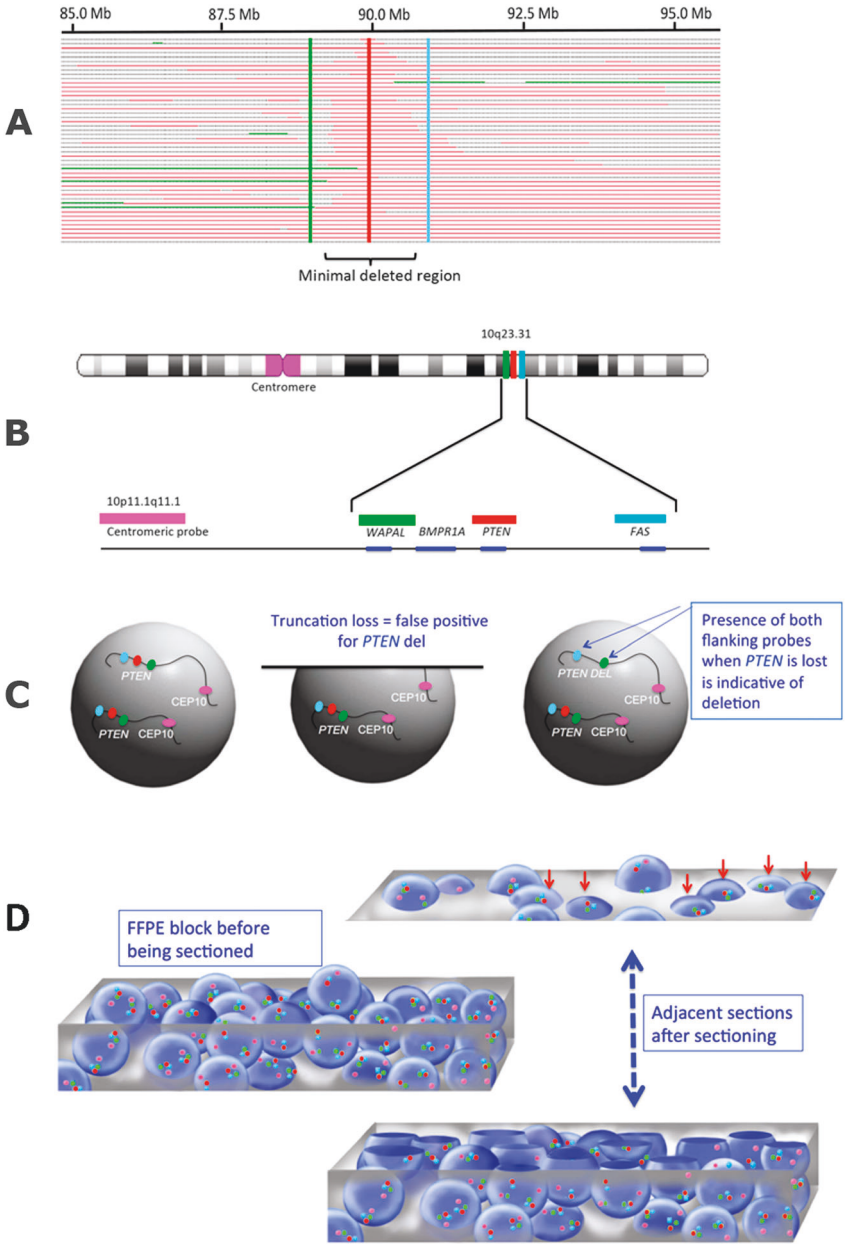

Fig. 1 Design of flanking probes to detect tumor suppressor gene deletions illustrated using an example of the *PTEN* gene. **a** Genomic deletion sizes of chromosome 10 derived from public domain datasets. Each horizontal thin red line represents an individual deletion and the best location for the selection of probes flanking *PTEN* (red vertical line) is just outside the minimal region of *PTEN* deletion (green and blue vertical lines). **b** Position of flanking probes *WAPAL* (green box) and *FAS* (aqua box) selected because they map outside the *PTEN* (red box) minimal region of deletion shown in **b**. The centromere probe (magenta box) is included to help determine the number of chromosome 10 s in the sample. **c** Schematic diagram to illustrate how a four-color probe design can be used to recognize truncation losses due to sectioning in a nucleus (gray spheres). In this illustration, the *PTEN* probe (red spots) is flanked either side of the gene by two probes (blue and green spots) on the two black wavy lines representing chromosome 10, which is enumerated by the CEP10 magenta probe. When there is loss of red *PTEN* with both blue and green flanking probes it indicates that sectioning (shown schematically as a dashed blue line in middle nucleus) has removed nuclear material that contained part of chromosome 10. However, when an actual interstitial deletion of *PTEN* is present, both flanking probes are retained (shown in right nucleus), indicating that the loss of a *PTEN* signal is due to an actual deletion. **d** Schematic three-dimensional representation to illustrate how four-color FISH is used to identify various truncated nuclei in a FFPE section. The nuclei are depicted as uniform pale blue spheres that are distributed randomly throughout the cuboid three-dimensional volume that represents part of a 5 µm section (left). In each nucleus the same spot colors as **a** are used to depict *PTEN* (red), the flanking probes (blue and green), and CEP10 (magenta). The thick blue dashed double arrowed vertical line points to the interface of two adjacent sections illustrating how truncated nuclei may be identified. It can be seen that there are six nuclei (red arrows) which will be recognizable with this probe set as truncated nuclei by the concurrent losses of a red *PTEN* probe with both green and blue flanking probes

FISH analysis using a four-color deletion assay

A newer approach to increase the specificity of FISH deletion assays is to utilize additional probes labeled with different colors that are positioned to allow sectioning artifacts to be recognized and excluded from the overall scoring. Three- and four-color FISH assays have been used in a number different diagnostic settings to increase the specificity for detecting the aberration of interest [25–28]. Multicolor deletion FISH assays usually incorporate a specific probe for detecting loss of the tumor suppressor gene, in combination with one or more control probes to determine whether signal truncation has taken place.

One of the best examples of four-color deletion FISH is the *PTEN* gene in prostate cancer [29]. We will use this tumor suppressor gene assay to illustrate the benefit of using additional control probes to identify nuclei that have been truncated by sectioning. The control probe combination was selected based on the idea that having adjacent probes that map either side of the *PTEN* tumor suppressor gene would allow for: (i) improved recognition of nuclear truncation losses; and (ii) would be helpful for mapping any larger deletions that may have extended outside the *PTEN* region. The best position for choosing flanking probes was found to be at the edge of the region of most frequent *PTEN* gene loss (Fig. 1a, b). When a nucleus loses both flanking probes together with the *PTEN* gene, there is a high chance that the nucleus was subject to truncation. In Fig. 1c, we show how the flanking probes are used to recognize a truncated nucleus as a schematic diagram. The concurrent loss of *PTEN* and both its flanking probes is a strong indication that the nucleus was sectioned during preparation. In Fig. 1d, the way that the flanking probes are used to recognize truncated nuclei in an analysis of part of a tissue section is depicted as a three-dimensional schematic representation.

Four-color FISH has been used to characterize the *PTEN* deletion status of prostatic carcinomas from both radical prostatectomy samples [29–31] and from needle core biopsies [32]. In these analyses, tumors in which there is no *PTEN* deletion (Fig. 2a) can readily be distinguished from truncated nuclei with *PTEN* losses caused by sectioning affects. For tumors with a homozygous deletion of *PTEN* (Fig. 2b) there is a complete absence of the *PTEN* specific

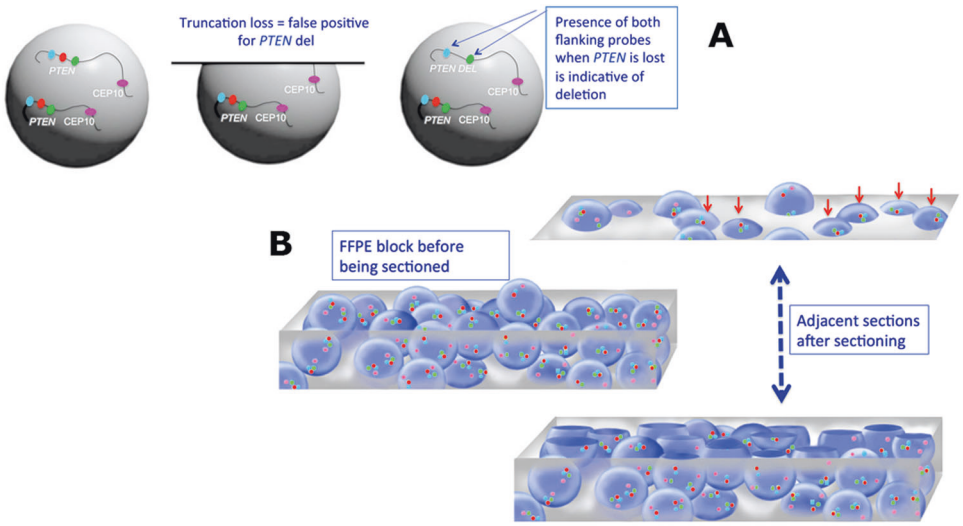

Fig. 2 Four-color detection of *PTEN* deletions in FFPE sections. Representative four-color FISH images of 5 µm sections from radical prostatectomy samples are used to show the typical distribution of red *PTEN* signals and to illustrate how the blue *FAS* flanking probe and green *WAPAL* flanking probes aid in the detection of truncated nuclei that could otherwise be misleading. In this figure and all other the *PTEN, FAS* and *WAPAL* probe are labeled with these same colors, and the *CEP10* probe is magenta (PTEN DEL-TECT Biocare, Pacheco, CA, USA). In all panels, the scorable nuclei without apparent loss of signal due to sectioning are indicated with blue arrows. Nuclei that are not scorable as a result sectioning affects are indicated with red arrows. One hundred nuclei were scored for each tumor to determine their respective *PTEN* deletion status. Scale bar, 2 µm. A schematic interpretation of the probe configuration on each chromosome 10 is shown beneath each panel. **a** Undeleted for *PTEN*. In the right panel, two red *PTEN* signals are apparent in all four scorable nuclei. However, two nuclei that have an apparent loss of *PTEN* can be recognized as being truncated (red arrows) as other probes are also lost with *PTEN*. **b** Homozygous *PTEN* deletion. In center panel, no *PTEN* signals are apparent in any nuclei. Some nuclei can be seen to be truncated (red arrows) because of loss of other probes. **c** Hemizygous *PTEN* deletion. In the right panel, the nuclei with blue arrows were scorable, and each has just one copy of the *PTEN* gene but retains flanking probes. The typical signal configuration shows seven signals with one red signal missing on all scorable nuclei. It is likely that this tumor has an interstitial deletion of chromosome 10 removing one copy of *PTEN*. This tumor is therefore classified as having a 'hemizygous deletion'. Truncated nuclei with other signal losses in other probes are identified by red arrows

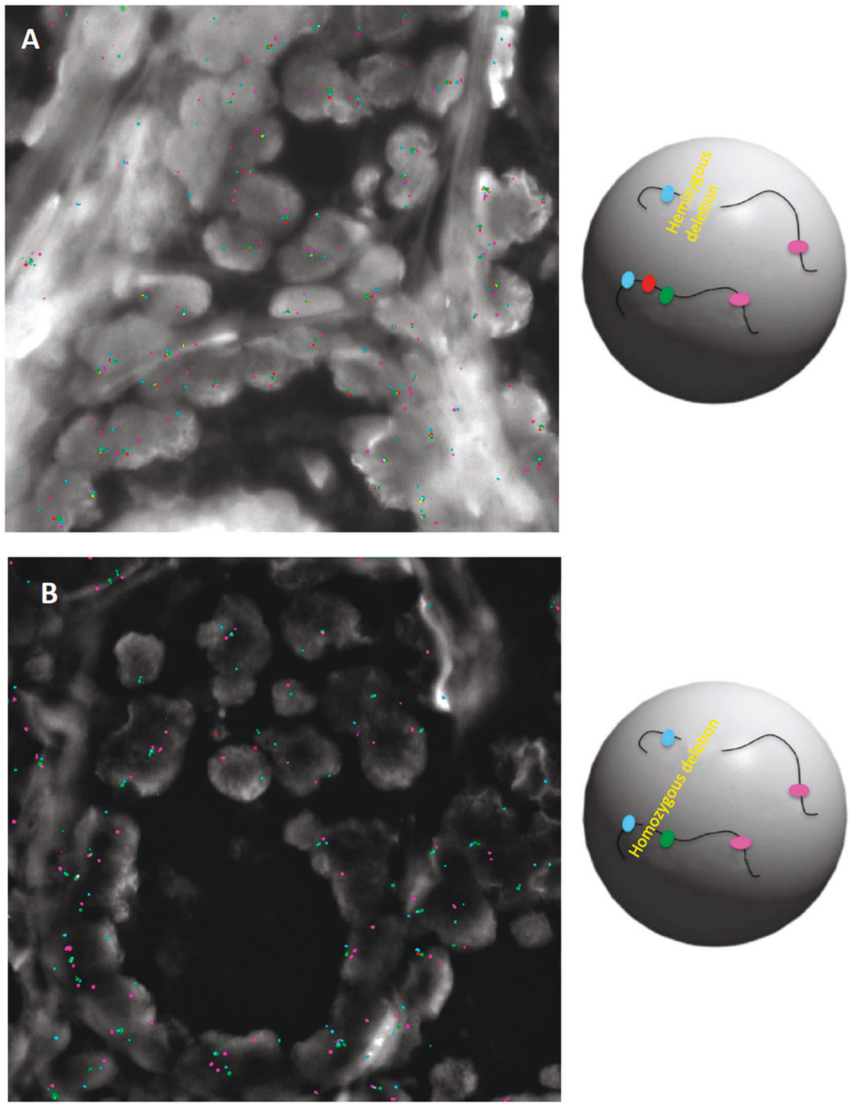

Fig. 3 Detection of clonally distinct hemizygous and homozygous deletions in different regions of a tumor. The four-color *PTEN* FISH probe set can help characterize genomic heterogeneity and variation in deletion size. In this example, one side of the prostate gland (upper panel A) had tumor cells with a large hemizygous *PTEN* deletion (red probe) that extended into the *WAPAL* green flanking probe (see the schematic interpretation of deletion on the right). On the other side of the gland (lower panel B) tumor cells had this same large deletion on one chromosome 10, but the other copy of the *PTEN* gene was also deleted, retaining both flanking probes. Thus the tumor cells in this region of the gland was homozygously deleted for *PTEN* with no red signals evident (see the schematic interpretation of both deletions to the right of this panel)

probe so that tumors with this aberration are relatively easy to interpret. Nevertheless, some truncated nuclei can be readily detected in sections as signal losses affecting the flanking probes and a centromere. The benefit of including flanking probes is more apparent for tumors with a hemizygous deletion in which only one copy of the *PTEN* gene is lost (Fig. 2c). Careful examination of nuclei in these cases shows that the predominant pattern in scorable tumor nuclei is cells with only one *PTEN* gene copy. In a few cells, nuclear truncation effects can lead to loss of both *PTEN* genes, but the concurrent loss of flanking probes with *PTEN* is used to minimize the risk of misclassifying a hemizygous tumor as being homozygously deleted.

Another advantage of a four-color *PTEN* FISH assays is that is possible characterize the genomic heterogeneity of deletions within different regions of carcinoma [30]. The flanking probes can be used to identify any clonal variations of deletion size in more detail within the tumor sections. For some prostate cancers, the occurrence of two distinct regions bearing hemizygous and homozygous clonal deletions has been used to identify heterogeneity of *PTEN* losses (Fig. 3).

Effect of section thickness on nuclear truncation losses

Nuclei in FFPE sections of cancers have a wide range of nuclear diameter. Prostate cancers typically have a mean diameter of about 6–10 µm [33, 34], and morphometric analysis has shown prostate cancer nuclei in FFPE sections often have an elliptical shape rather than being perfect spheres [35]. FISH signals are usually small discrete signals distributed within the nuclear space. To investigate the

three-dimensional appearance and the spatial distribution of two-colored FISH signals within nuclei of tumor sections we performed high-resolution confocal imaging using FFPE prostate cancer sections. Three-dimensional views were created by building stacks of each optical slice collected at sequential Z-axis locations (Supplementary Movie 1). This analysis showed that the FISH signals were present randomly at different depths within the nuclear space and that some nuclei were partially present in the section and had a reduced number of FISH signals. We then captured images at different focal planes through a 5 µm prostate cancer section (which was not deleted for *PTEN*) to show how the *PTEN* and flanking probe FISH signals were distributed within nuclei and the effects of signal truncation (Supplementary Movie 2).

Early theoretical studies of the general effect of section thickness on the loss of FISH signals by truncation have also shown that when the thickness is <50% of the mean nuclear diameter the proportion of truncation losses increases [36]. We have estimated that the theoretical effect of reducing section thickness from 5 to 3 µm would increase the number of signal losses due to truncation by ~20% (Supplementary Material 1).

To directly investigate the influence of section thickness and truncation of nuclei on scoring results, we compared the FISH results of the four-color *PTEN* probe set on 3 µm FFPE tissue sections (Fig. 4a) to signal counts obtained from 5 µm FFPE normal tissue sections (Fig. 4b). Slides were evaluated by conventional fluorescence microscopy in the same regions previously defined and marked on each slide. These results showed that, while the percentage of signal truncations leading to potential misclassification of *PTEN* as a hemizygous loss was 11% in 5 µm sections, this frequency was much higher in 3 µm sections, with 37% having signal truncations leading to the false classification of *PTEN* deletion (Supplementary Material 2). Similarly when a *PTEN* hemizygously deleted tumor was analyzed using a 5 µm section, only 5% of cells could have been misclassified as being homozygously deleted without the use of flanking probes. This frequency of misclassification would have increased to 13% in the thinner 3 µm sections if the flanking probes had not been used to recognize signal truncation artifacts.

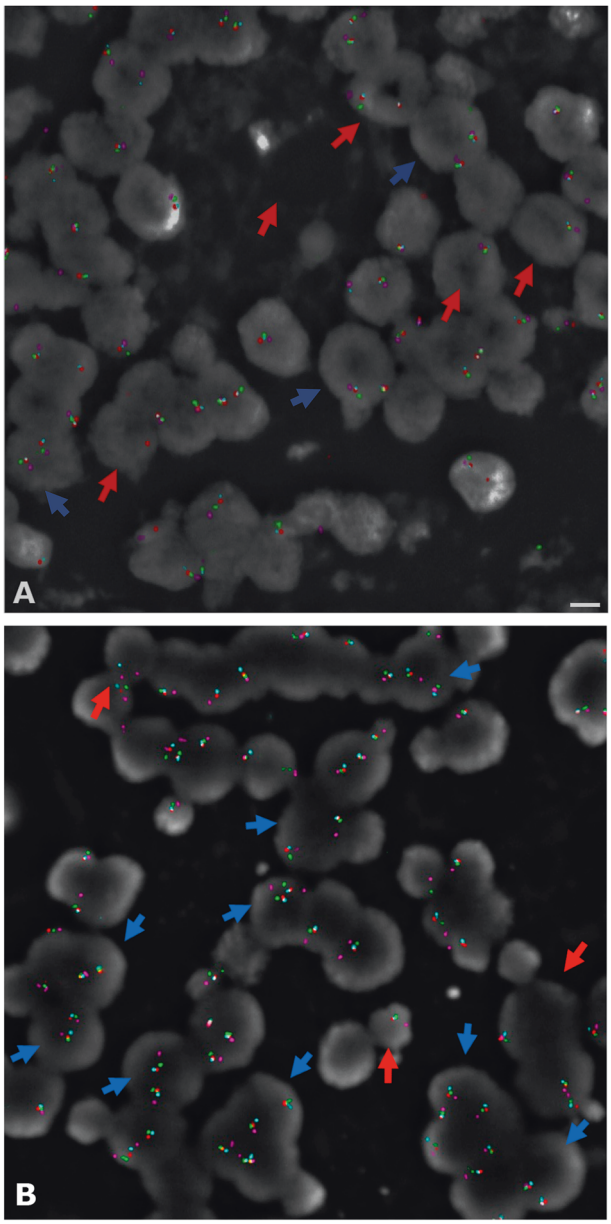

Fig. 4 Effect of section thickness on nuclear truncation. The four-color *PTEN* FISH probe set was used to compare the relative amount of nuclear truncation when 3 µm (upper panel A) sections were compared to 5 µm (lower panel B) sections. In this comparison, we used one prostate carcinoma without an apparent *PTEN* deletion or other genomic alteration affecting the copy number of chromosome 10. In both panels, the scorable nuclei without apparent loss of signal due to sectioning are indicated with blue arrows. Nuclei that are not scorable as a result sectioning effects are indicated with red arrows. One hundred nuclei were scored for both 3 and 5 µm sections to determine their respective frequencies of nuclear truncation. Scale bar, 2 µm

Guiding principles for interpretation of FISH deletion assays

Tissue sections derived from solid tumors present a complex and challenging target for FISH assays. The distribution of nuclei within the section space is irregular, determined by multiple factors including the architecture of the tissue, as well as normal histologic variables such as differing levels of the intermingled tumor and adjacent stroma. In addition to these general histologic features, the nuclei within the tumor tissue vary in size as a function of their overall cell shape, their ploidy level, and the degree of local nuclear compaction present in the preparation. In Table 2, we present some of the general principles to

Table 2 General guidelines for FISH deletion assays using FFPE tissue sections

FISH test validation	It should be performed according to the most appropriate Standards and Guidelines for Laboratories [22–24, 40, 41], and for monitoring and reporting data [42]. There are a number of reviews that address clinical applications of FISH [43, 44].
Standard controls	The laboratory should periodically check assay performance (including control probes) as part of quality monitoring. Monitoring FISH testing over time to assess adverse technical trends is also recommended.
Analytical standards	Assessment of several normal metaphase cells should be considered for validation that the correct probe was used for the study: • In typical analytic validations, the FISH probe is hybridized to metaphase and interphase cells from peripheral blood cultures of five karyotypically normal control males. • For each specimen, the number of FISH signals in at least 50 consecutive interphase cells is recorded, and then the hybridization sites in 20 metaphase cells are identified by banded chromosome morphology. • The analytic sensitivity and specificity for metaphase cells, and the percentage of nuclei that meet the signal pattern criteria for normal cells are calculated as described [23]. • This evaluation also ensures that there are no background signals or cross-hybridizations to related genes that could be misinterpreted in interphase FISH tissue section analyses.
Establishment of cutoff values	The cutoff levels to be used to identify a sample as deleted should be established as part of the FISH test validation for the laboratory. • The cutoff value used is established by analyzing a reference panel of histological tissue sections from normal healthy cases. • The use of suitable normal control tissue with similar sized nucleus to the target tissue being analyzed can help to establish the expected percentage of signal losses due to signal truncation artefacts. In this context, setting up a normal database for each probe being used in the laboratory is suggested. • The laboratory's cutoff database should address each type of target tissue and it should identify the thickness of samples used for FISH (the same thickness should be maintained for all specimen testing). Wiktor et al. [23] published an excellent clinical FISH validation approach, which describes a cutoff method to establish an analytic sensitivity with a 95% confidence level. The ECA guidelines also discuss various approaches to establishing robust cut-offs [40]. • Monitoring and revising existing cutoff values should also be considered as probes used and test approaches change with time.
Positive and negative controls	If possible run positive and negative controls. For FISH deletion assays these will be samples with known homozygous, hemizygous and undeleted copies of the tumor suppressor gene of interest.
Tissue section thickness	The standard thickness of unstained tissue sections is between 5 - 6 microns. The tissue sections must be applied to a positively charged or silanized slide to minimize the incidence of tissue sections falling off the slides during processing. Tissue sections less than 5 - 6 microns will result in an increased truncation of nuclei affecting established cutoff values, and a greater thickness will result in poor probe hybridization.
Hematoxylin–eosin slides	An hematoxylin–eosin slide must accompany unstained slides for FISH test with the diseased area circled in either pen or felt marker. Designated areas should be representative of viable tumor regions, with necrosis, hemorrhage, poor fixation, or histological artifacts being excluded.
General considerations for FFPE FISH analysis	Use optimal filter set for the deletion assay probe combination and check there is no bleed-through between different filters. Review slides for hybridization performance. There should be >85% efficiency with minimal non-specific noise. Pre-screen the tumor area selected by the pathologist using an adjacent hematoxylin and eosin section map for the following features: • The area is tumor rich • Nuclei have a regular shape and uniform DAPI staining • Nuclei do not have evidence of digestion damage such as 'doughnut- like' appearance with empty epicenters • Nuclei should not be covered by a cloudy typically yellowish layer or obscured by auto-fluorescent structures. • Nuclei have hybridization signals with uniform intensity and similar patterns of granularity.

Table 2 (continued)

	Preparations not meeting these criteria should not be used for signal enumeration.
	Ensure that the entire selected area of tumor has been pre-screened carefully before selecting nuclei to score. Sometimes a small area containing a clonal deletion may be missed without this pre-screen.
	Only examine nuclei that are distinct and ideally separated from each other. Select cells in which the borders of individual nuclei can be clearly distinguished. Avoid scoring nuclei that are crowded, overlapping, or distorted.
	When selecting nuclei focus up and down on the z-axis and make sure the entire volume of the chosen nucleus is present inside the section and that the FISH signals at all focal planes are enumerated. A bias in distribution to the upper or lower face of the section may indicate truncation.
	Signals may be either bright and compact oval shapes, split into two smaller but connected dots, or a stringy diffuse shape. Pay attention to the signal intensity.
	The probes flanking the tumor suppressor gene can help distinguish between truncation losses and 'real' interstitial deletions. Sometime the deletion may be larger and include one (or both) flanking probe sets.
Scoring of probe signals	All scores should be entered onto score sheets in an unbiased manner. A routine FISH evaluation should be scored by two technologists.
	All scores should be entered onto score sheets together with comments that may be relevant concerning heterogeneity, signal quality etc.
	Score appropriate number of nuclei according to the Standards and Guidelines for Laboratories. When inconsistent results are obtained a third reader is required or additional nuclei should be scored based on the laboratory director's guidance.
	Be aware of the possibility of clonality of deletions (such as mixture of hemi-and homozygous deletion). All clones should be evident once appropriate number of nuclei has been scored.
	Sometimes in complex cases there is more than one type of clone:
	• Each clone should be scored individually (score appropriate number of nuclei for each clone) and the location of the clone marked on the hematoxylin and eosin section map.
	• In such complex tissue where there is more than one type of aberration, each clone should be scored individually (ideally scoring 100 cells for each).
	Once completed the scoring, re-scan the marked tumor area to ensure nothing has been missed.
	Typical scoring results for tumor suppressor gene FISH assays will describe the % of normal cells, the frequency of homozygous and/or hemizygous deletions or monosomies. In addition, there may be a percentage of cells with ploidy alterations or gains of the chromosome.
	The criteria for scoring deletion FISH should in general be developed for the tumor suppressor gene of interest after taking into account the previous experience of the laboratory and using data from the literature from other groups performing similar assays.
Quality control	Ensure that signals from all probes are present in normal surrounding tissue adjacent to tumor areas on all slides to confirm successful FISH hybridization.

consider when examining FFPE tissue sections using any deletion FISH assay.

Future of clinical deletion assays of solid tumors

This review has focused primarily on recent improvements for FISH testing in clinical laboratories to detect deletions of tumor suppressor genes in a solid tumor using FFPE tissue sections. However, newer 'next-generation sequencing (NGS)' and sequence-based technologies are providing alternative approaches to detecting both changes in gene copy number, and somatic point mutations [37]. NGS approaches can be optimized for analyzing DNA derived from tumor specimens that have been fixed in formalin [38]. These assays typically use methods such as multiplex PCR to isolate clinically relevant DNA segments of the genome, such as mutation hotspots or coding exons of entire oncogenes or tumor suppressor genes [39]. Counting the number of sequencing reads that align to a given genomic location is analogous to enumerating interphase FISH signals for a specific gene probe, but at extremely high resolution. One of the main general limitations of sequence-based methods for deletion detection is that often a solid tumor specimen contains significant infiltrations of normal stromal and inflammatory cells that may reduce the sensitivity of DNA copy-number measurements or make copy-number counting

inconclusive. Future assessment of deletions for clinical needs will likely be based on combining findings from FISH, immunohistochemistry with customized NGS sequence-based detection methods.

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Compliance with ethical standards

Conflict of interest JAS has consulted for CymoGenDx LLC, and MY and JAS have a provisional patent application on probe sets to detect deletions

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