Out of the darkness and into the light: bright field in situ hybridisation for delineation of *ERBB2* (*HER2*) status in breast carcinoma

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ABSTRACT

Assessment of *ERBB2* (*HER2)* status in breast carcinomas has become critical in determining response to the humanised monoclonal antibody trastuzumab. The current joint College of American Pathologists and the American Society of Clinical Oncology guidelines for the evaluation of *HER2* status in breast carcinoma involve testing by immunohistochemistry and fluorescence in situ hybridisation (FISH). However, neither of these modalities is without limitations. Novel bright field in situ hybridisation techniques continue to provide viable alternatives to FISH testing. While these techniques are not limited to evaluation of the *HER2* gene, the extensive number of studies comparing bright field in situ techniques with other methods of assessing *HER2* status allow a robust evaluation of this approach. Analysis of the literature demonstrates that, when used to assess *HER2* gene status, bright field in situ hybridisation demonstrates excellent concordance with FISH results. The average percentage agreement in an informal analysis of studies comparing *HER2* amplification by chromogenic in situ hybridisation with FISH was 96% (SD 4%); κ coefficients ranged from 0.76 to 1.0. Although a much smaller number of studies are available for review, similar levels of concordance have been reported in studies comparing *HER2* amplification by methods employing metallography (silver in situ hybridisation) with FISH. A summary of the advancements in bright field in situ hybridisation, with focus on those techniques with clinical applications of interest to the practicing pathologist, is presented.

INTRODUCTION

Historical perspectives on in situ hybridisation

At the time of Watson and Crick's published description of DNA structure in 1953,[1] Tjio and Levan had yet to publish the first reliable determination of the normal human chromosome complement.[2] Much discovery was still needed before early knowledge of DNA technology could be applied to the field of cytogenetics.[3] Although the technique of DNA–DNA hybridisation had been introduced in 1961,[4] it was not until 1969 that successful attempts using radiographically labelled DNA and RNA to identify chromosomal targets of cytological preparations were made.[5 6] These early studies relied upon a tritium-labelled RNA probe, derived from mixtures of *Xenopus* 28S and 18S RNA, and alkaline denaturation of extrachromosomal rDNA from *Xenopus* oocytes.[5] Hybridised sequences were detected by autoradiography. Although limited by the resolution of the radiographic detection method employed, Gall and Pardue were able to demonstrate that RNA, and soon after DNA, can be hybridised specifically to target sequences under conditions that 'preserve the morphological integrity of the nucleus'.[5 6] Furthermore, the ability of this in situ technology to quantify relative amounts of target sequence was suggested by the detection of a low level gene amplification in premeiotic oogonia.[5] Additional successes were soon reported in employing autoradiographic detection of rRNA and DNA hybrids in tissue sections and in cytological specimens.[7 8]

Over the years, much improvement has been made in the processes with which probes are developed and labeled, including the introduction of random primer labelling, nick translation reaction and PCR-based labelling.[3] Revolutionary discoveries were reported in 1982 by two groups who performed hybridisation experiments with probes labelled either fluorimetrically or cytochemically, rather than with radioisotopes.[9 10] These fluorescent labels provided many advantages to the in situ hybridisation technique, including improvements in the easy and safety of use, increases in resolution, and the possibilities of simultaneously identifying multiple targets within the same nucleus.[11] This new technique, fluorescence in situ hybridisation (FISH), could be accomplished using a probe labelled either directly or indirectly with a fluorochrome, and the basic principles of these labelling techniques have been recently reviewed.[12] Briefly, direct labelling is the process of incorporating fluorescently labelled nucleotides into the nucleic acid probe; indirect labelling often involves complexing the probe with an intermediary hapten (eg, digoxigenin) that is subsequently detected with a labelled antibody to identify the target sequence of interest.

By 1985, another milestone in the in situ hybridisation technique was achieved when Landegent *et al* demonstrated localisation of the human thyroglobulin gene to a specific chromosome band using a probe constructed from cosmid subclones of the 3′ region of the thyroglobulin gene.[13] By the turn of the century, further refinement of the FISH technique lead to routine localisation of DNA targets as small as 10 kb and the ability to localise segments as small as 1 kb.[11] Technical advancements through the years have spawned a variety of FISH technologies,[14] and many of these experimental achievements are considered among the most significant milestones in the field of cytogenetics and molecular pathology.[3] FISH has been particularly successful for mapping single-copy and repetitive DNA sequences using metaphase and

interphase nuclei, for detecting targeted chromosome translocations, and for localising large repeat families to aid in chromosome identification and karyotype analysis. The research application of this technology is vast; clinically, FISH has proved invaluable in the diagnosis, prognostication and pharmacogenomic assessment of many diseases.

Despite the advantages of FISH, the technique is not without drawbacks. Often cited limitations to the routine implementation of conventional FISH include the requirements of a dedicated fluorescence imaging system and well-trained personnel with specific expertise. Furthermore, FISH studies provide relatively limited morphological assessment of overall histology, reduced stability of the fluorescent detection signal(s), and overall higher cost of testing. These limitations have prompted new achievements in the arena of in situ hybridisation detection. The purpose of this review is to summarise the advancements in bright field in situ hybridisation in use today with a focus on those techniques with clinical applications of interest to the practicing pathologist.

Clinical applications of bright field in situ hybridisation: the *HER2* story and beyond

The continuous evolution of our understanding of the molecular pathogenesis of disease is perpetually altering our clinical decision making and therapeutic strategies. These changes have placed pressure upon clinical laboratories to provide adequate testing platforms to provide insight into the status of the disease of an individual patient. For many neoplastic processes, tissue microscopic morphology is the foundation to a diagnosis being made, and paraffin-embedded tissue provides an abundant source of archived material for molecular testing. As the need for molecular testing has increased, multiple techniques have been created or incorporated into the clinical laboratory to provide these necessary results. The various in situ hybridisation techniques meld a focused genetic technique upon the histology slide platform. Nonetheless, the amount of architectural information available for review depends on the type of in situ hybridisation procedure used. Bright field in situ hybridisation is particularly beneficial in this regard, as the majority of the morphological detail present on routine H&E-stained sections is preserved. Although in situ hybridisation can be used to assess a myriad of different molecular genetic aberrations,[15] investigation of the *HER2* gene status in breast carcinoma has been a major impetus for the development of bright field in situ hybridisation techniques.

As one of the five leading causes of cancer deaths worldwide, The World Health Organization recently projected that breast cancer will cause 630 000 deaths in 2015.[16] This disease burden necessitates efficient use of limited healthcare resources. The ability to target specific genetic aberrations that are susceptible to a specific therapy is becoming a best clinical practice for treating a variety of diseases, particularly neoplasia. Discovery of the role of *HER2* in breast cancer, and subsequent discovery of a viable corresponding gene-specific therapy, highlights the central role of a specific genetic aberration in some breast cancers, the ability to create therapeutics that target these specific aberrations, and the crucial necessity to identify the molecular genetic status of an individual's breast cancer to personalise the clinical management. The experience with *HER2* in breast carcinoma exemplifies the melding of a specific laboratory test with a specific therapy, pharmacogenomics, and the important role of in situ technology in clinical practice.

HER2 (*ERBB2*) is a proto-oncogene that encodes for a 185 kDa protein that is a member of the ERB family of transmembrane tyrosine kinase receptors. This receptor exists in a dimerisation-ready conformation, and does not require ligand binding to form functional dimers. Although it can form homodimers, it rarely does. Rather, it preferentially forms heterodimers with the remaining members of its family, particularly *HER3*. Depending upon the heterodimer, various signalling pathways are activated.[17] This results in *HER2* playing a role in different cellular functions, including the promotion of cell division and survival, while inhibiting apoptosis. These various functions reflect its potential to produce an oncogenic effect following *HER2* gene amplification.

In 1987, Slamon *et al* published the first study identifying the role of *HER2* in a subset of breast cancers.[18] The authors demonstrated that *HER2* amplification by Southern blotting was an independent variable linked to inferior overall survival and progression-free survival in multivariate analysis. During this same time period, Greene *et al* provided evidence that monoclonal antibodies against the p185 product of *HER2* inhibited *HER2*-transformed cell lines implanted in nude mice.[19] These studies, among others, laid the foundation for the development of a targeted therapy in breast cancer: trastuzumab.

Trastuzumab, or Herceptin, is a humanised monoclonal antibody against the 185 kDa protein of *HER2*. Its effect upon *HER2*-positive breast cancer is not limited to the immune response upon antibody binding. Rather, its effects are diverse and include the inhibition of dimerisation, induction of apoptosis, decreased cellular proliferation, and the modulation of signal transduction pathways. In 2001, the first phase III clinical trial of trastuzumab was published by Slamon *et al*.[20] This prospective study examined the effect of trastuzumab on overall and progression-free survival in a cohort of women with metastatic breast cancer. A significant improvement in progression-free survival was demonstrated when trastuzumab was added to the chemotherapeutic protocols. Later studies, with a longer follow-up period, confirmed a significant improvement in overall survival when trastuzumab was added to the treatment of women with metastatic breast cancer. In 2007, the HERA international multicentre randomised trial reported on the use of trastuzumab in patients with *HER2*-amplified early stage breast cancers.[21] A 2-year follow-up of the study showed a significant improvement in overall survival when trastuzumab was used in conjunction with standard therapeutic regimens. Currently, trastuzumab is used in the adjuvant setting for treatment of early stage breast cancer as well as metastatic breast cancer. Although the role of trastuzumab as neoadjuvant therapy is still being investigated,[22] preliminary studies have demonstrated a significantly better pathological complete response in patients receiving neoadjuvant trastuzumab in combination with other agents.[23–25]

Throughout these studies, the drug toxicity of trastuzumab has been a concern, particularly the cardiac side effects.[26] These ranged from mild left ventricular dysfunction to severe congestive heart failure. The severity of side effects further emphasised the clinical imperative to use trastuzumab only in the subset of patients whose clinical benefit would outweigh the risk of treatment side effects.

The clinical utility of trastuzumab, juxtaposed with the potential for drug toxicity, mandates the use of this therapy in the select group of patients who demonstrate *HER2* amplification. This creates a clinical laboratory imperative to provide accurate and precise testing when assessing the *HER2* status in breast cancer patients. In 2007, the American Society for Clinical Oncology and the College of American Pathologists (ASCO/CAP) published a joint guideline to standardise *HER2* testing in the USA.[27 28] The panel provided testing algorithms and test interpretation guidelines. The concern of equivocal or false positive results by immunohistochemistry is illustrated in the

interpretation guidelines. The panel redefined 2+ immunohistochemical staining for the *HER2* gene product as equivocal, rather than positive. This change reflects two features previously identified of this category of test results. First, a large portion of cases that stain 2+ fail to show gene amplification by FISH. Considering that FISH has a high concordance with Southern blotting, it was decided that 2+ scoring was equivocal with regards to *HER2* status rather than positive. Second, up to 15% of clinical cases assessed using immunohistochemistry (IHC) fall within the equivocal category. Their recommendation requires that cases that are equivocal by IHC be retested using a validated assay for the *HER2* gene status. Specific guidelines for interpretation of bright field in situ hybridisation results were also provided by the panel.

These pressures provide impetus to further develop laboratory tests to fulfil this testing requirement. Although an armamentarium of strategies to detect *HER2* status in research and clinical laboratories exists, including Southern blotting, PCR, IHC and FISH, the limitations of each of these have been documented.[29] More specifically, although FISH is a robust test, its complicated procedure coupled with high technical expertise requirements precludes its use except in laboratories equipped and staffed to perform and interpret this highly complex testing. Over the last decade, the development of bright field in situ hybridisation techniques attempts to address the difficulties limiting widespread FISH testing.

Although bright field in situ hybridisation has gained much attention through the need to develop an accurate test of *HER2* status that can be performed efficiently and cost effectively in many clinical laboratories, the application of this technology is not confined to assessment of *HER2* status in breast carcinomas. Several other gene targets have been under investigation, and the implications of testing for these have been reported.[30–34] Recently, published studies have used chromogenic in situ hybridisation as a means of assessing platelet-derived growth factor receptor A in gliomas,[35] determining amplification of the epidermal growth factor receptor (*EGFR*) gene in anal squamous lesions,[36] and correlating the *EGFR* gene copy number with therapy response in colorectal cancers.[37] Furthermore, the use of silver-enhanced in situ hybridisation to evaluate *EGFR* status in human glioblastomas has demonstrated strong concordance with FISH and gene expression data.[38] The principles of various bright field technologies in use today, along with their benefits and limitations, are described below.

A NEW DAWN APPROACHES

Chromogenic in situ hybridisation

The basic principles of in situ hybridisation are straight forward and can be simplified as: use of a DNA probe complementary to a target sequence of interest followed by detection of the bound probe.[39] Generation of the probe, method of labelling, condition of hybridisation, and strategy for detection are all areas of nuance that depend upon the type of in situ hybridisation technique employed.[40] Chromogenic in situ hybridisation (CISH) was first described by Tanner *et al* in 2000 as an alternative to FISH detection of *HER2* amplification in archival breast tissue.[41] In that study, paraffin-embedded tissue sections were pretreated and subsequently hybridised with a digoxigenin-labelled DNA probe. The probe was detected by use of antidigoxigenin fluorescein, followed by antifluorescein peroxidase and diaminobenzidine. The basic principles of this CISH technique are outlined in figure 1.

Although enzymatic DNA in situ hybridisation of *CCNDI* had been previously described, the study by Tanner *et al* was the first

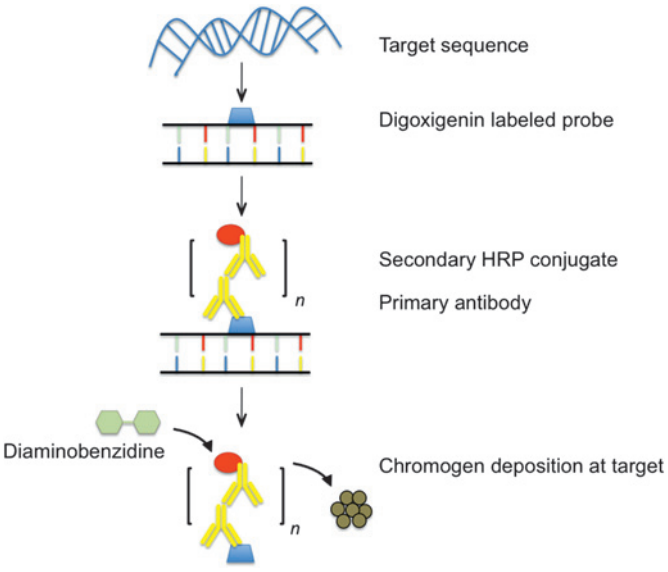

Figure 1 Conceptual schematic of single-colour chromogenic in situ hybridisation demonstrating bright field detection of a digoxigenin-labelled probe. The probe is recognised by an antidigoxigenin fluorescein isothiocyanate primary antibody followed by detection with an anti-fluorescein-isothiocyanate horseradish peroxidase (HRP). After addition and oxidation of diaminobenzidine, a dark brown signal is deposited at the target site.

to examine the status of the *HER2* gene in paraffin-embedded tumour samples using a modified detection system with superior sensitivity to the antidigoxigenin plus biotynlated-tyramine amplification, with visualisation using diaminobenzidine and hydrogen peroxide, already in use.[42] While improvements upon the use of cosmid, P1, PAC and BAC clone probes for bright field in situ hybridisation had been reported,[43] additional advances were made in the CISH technique through pretreatment of tissue sections by heating in a microwave followed by a short period of enzyme digestion. The detection system used was an anti-digoxigenin-fluorescein isothiocyanate antibody plus an anti-fluorescein-isothiocyanate horseradish peroxidase conjugate.[41] This original *HER2* CISH procedure involved single-colour detection of one probe, similar to the US Food and Drug Administration (FDA) approved FISH testing for *HER2* available at the time. Comparison of CISH detection of *HER2* to that of FISH correlated well in the series of 157 breast cancers examined (93.6% concordance).[41]

Since the study by Tanner *et al*, additional variations of CISH technology have been evaluated. In general, the CISH technique employs either antibodies or other proteins (eg, avidin) conjugated to an enzyme (eg, horseradish peroxidase) in order to produce a chromogenic, rather than a fluorometric, reaction. Unlike FISH, chromogenic in situ hybridisation performs best when indirect labelling of the probe is used.[12] Examples of the staining quality that is achievable through the CISH technique are demonstrated in figure 2. Although a large variety of commercial probes are available for testing by FISH, a relatively limited number of probes are available for CISH.[12] However, reliable protocols to generate probes for chromogenic and fluorescence in situ hybridisation have been described.[44]

Our informal analysis comparing CISH with FISH for detection of the *HER2* gene in breast cancers demonstrates that the average percentage agreement in the examined studies comparing *HER2* amplification by CISH and FISH is 96% (SD=4%) (table 1). Although not always performed, κ coefficients ranged from 0.76 to

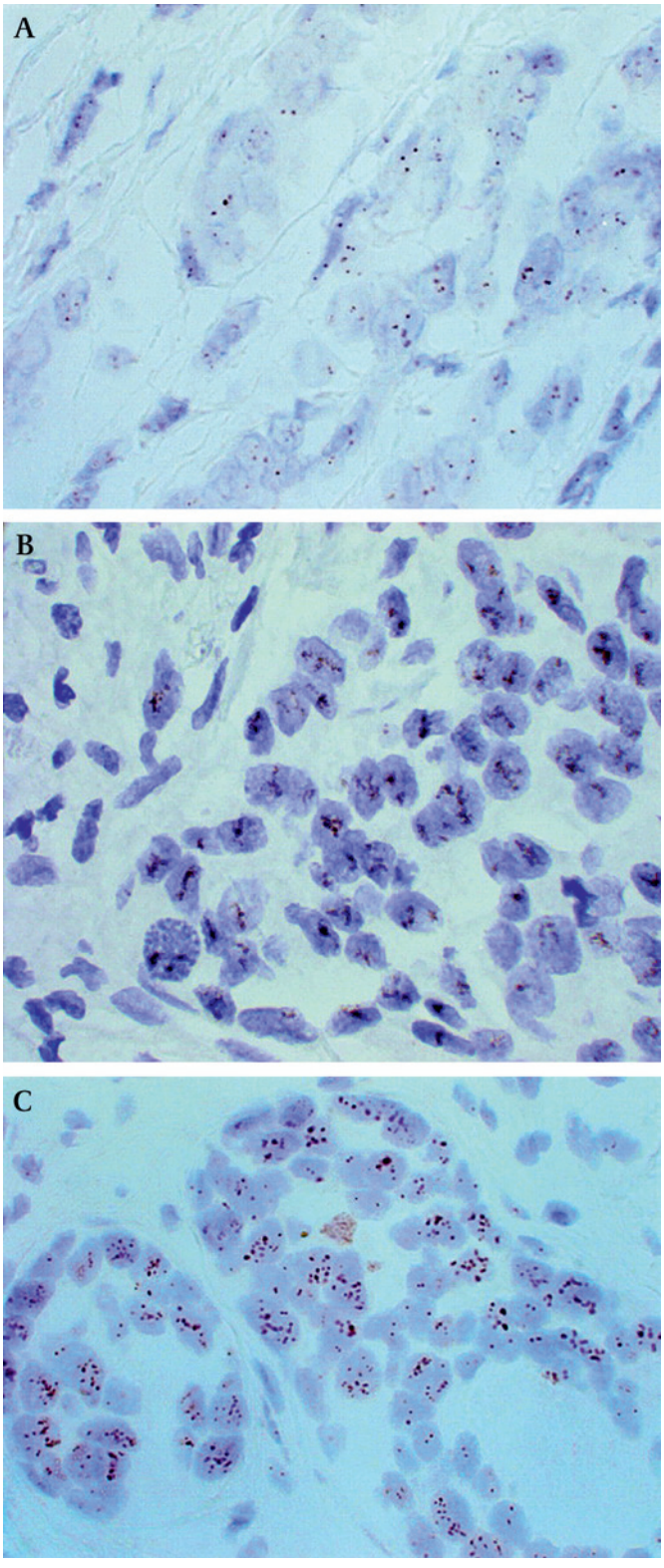

Figure 2 Examples of *HER2* detection using the chromogenic in situ hybridisation technique in breast carcinoma. (A) Demonstration of non-amplified *HER2* producing 1–2 signals per nucleus. Examples of *HER2* amplification where the peroxidase signal exists as either a cluster of gene copies (B) or as multiple individual gene copies (C). Original magnification ×600. Reproduced from Tanner *et al*[41] with permission from the American Society for Investigative Pathology.

1.0 with the exception of one study.[61] Of the studies reviewed, the findings from several larger studies warrant additional attention.

In 2004, Isola *et al* reported a study of paired CISH/FISH results from 192 breast cancers.[50] Similar to previously published studies, the authors demonstrated excellent concordance between CISH and FISH (93.8%), κ coefficient 0.875. After careful analysis of 12 cases that displayed discordance between the two testing modalities, it was determined that 7 of the 12 could have been resolved by having chromosome 17 information when performing the CISH evaluation, and the other five discrepant cases were due to histological features that were difficult to identify in the sampled tumour. The authors concluded that CISH is an accurate and feasible alternative to the FDA-approved FISH test (Vysis PathVysion; Abbott Molecular, Des Plaines, Illinois, USA).

In 2006, two groups independently published studies comparing *HER2* status determined by FISH and CISH.[57 58] The two studies analysed 200 or more breast cancers. Hanna and Kwok examined tumour samples by CISH and FISH in three groups based upon *HER2* status, as determined by IHC. Out of groups with either 0/1+ or 3+ staining by IHC, concordance between CISH and FISH was 97% and 98% respectively. Only 3 of 119 cases demonstrated discordance by these two methods. From the group with 2+ staining by IHC methodology, 9 of 135 cases demonstrated discordance between CISH and FISH (93% concordance). Discordant cases were of tumours displaying very low or borderline amplification with FISH. Scoring of samples with low-level FISH amplification is known to be difficult due in part to the high level of interobserver variability with these samples.[59] Overall, the authors conclude that evaluation of *HER2* by CISH may be a viable alternative to FISH analysis in the testing algorithm.

Saez *et al* examined 200 cases of invasive breast cancer to compare the status of *HER2* as determined by CISH and FISH.[57] The examined breast cancer cases were routinely examined by IHC, and during a 4-year period 95 cases with 0/1+ staining, 43 cases with 2+ staining, and 62 cases with 3+ staining, were collected for the study. A tissue macroarray of these cases was generated, and 174 of the cases were available for evaluation by CISH and FISH. Overall, a concordance of 94.8% was found between CISH and FISH. Of discordant cases, only one was identified as amplified by FISH and not by CISH. Eight breast cancers demonstrated amplification by CISH (two cases with low level amplification), but no amplification by the FISH technique.

In 2007, van de Vijver *et al* published an international validation ring study involving five pathology laboratories who undertook CISH assessment of *HER2* in breast cancer cases.[59] A total of 211 invasive breast carcinomas were analysed by CISH, and the results compared with data generated by FISH testing. Of the 76 cases with high levels of *HER2* amplification (HER2/CEP17 ratio >4), 96% tested positive for amplification by CISH. A concordance rate of 94% was achieved when testing 100 FISH-negative cases. However, in cases with low-level *HER2* amplification by FISH (HER2/CEP17 ratio 2.0–4.0), only a 57% concordance rate was achieved (20/35 CISH scores indicated amplification). In addition to the difficultly in assessing low amplification cases by FISH, part of this discordance was thought to be due to assessment of only the *HER2* locus by CISH, without normalisation for the chromosome 17 copy number in tumour samples. Although these cases pose difficulty for evaluation by CISH alone, it was proposed that counting signals from additional cells and using an additional CISH probe for chromosome 17 on an additional slide would be helpful. Even though it was estimated that the number of clinical breast cancers that fall into the category of borderline amplification of *HER2* by FISH is 1–3%,[59] practical solutions to the level of discordance in the

Table 1 Comparison of *HER2* status using CISH and FISH methodologies

Reference	Sample size	No. of test sites	Concordance (%)†	κ Coefficient*
Tanner *et al*[41]	157	1	93.6	0.81
Zhao *et al*[45]	62	1	100.0	NS
Dandachi *et al*[46]	38	1	100.0	NS
Gupta *et al*[47]	31	2	83.9	NS
Park *et al*[48]	188	1	94.1	0.84
Arnould *et al*[49]	75	8	96.0	0.97
Isola *et al*[50]	192	2	93.8	0.88
Hauser-Kronberger *et al*[51]	38	1	100.0	NS
Bhargava *et al*[52]	102	1	100.0	NS
Gong *et al*[53]	80	1	95.0	0.85–0.91
Lin *et al*[54]	25	1	92.0	NS
Li-Ning-T *et al*[55]	32	1	96.9	NS
Loring *et al*[56]	110	1	99.0	NS
Saez *et al*[57]	174	1	94.8	0.86
Hanna and Kwok[58]	254	1	95.1	0.91
van de Vijver *et al*[59]	211	5	88.6	NS
Cayre *et al*[60]	55	1	91.5	0.76–0.88
Sinczak-Kuta *et al*[61]	55	1	NS	0.53
Di Palma *et al*[62]	161	1	100.0	NS
Carbone *et al*[63]	89	5	98.9	NS
Di Palma *et al*[64]	28	7	98.5	0.91
Pothos *et al*[65]	88	1	100.0	NS
Gong *et al*[66]	226	2	98.8	0.93–1.0
Pedersen and Rasmussen[67]	72	1	98.6	0.97

CISH, chromogenic in situ hybridisation; FISH, fluorescence in situ hybridisation; NS, not specified.
*95% confidence level unless otherwise specified, coefficients rounded to two decimal places.
†Weighted averages were calculated in some instances.

van de Vijer study are needed. The overall conclusion of the study by van de Vijer *et al* was that CISH and FISH have very high concordance, and that CISH is a viable alternative to FISH for assessment of *HER2* in breast cancer cases.

The study by van de Vijer *et al* was not the first to identify that assessment and interpretation of *HER2* cases with very low level of amplification (6–10 signals per cell) benefit from inclusion of the chromosome 17 probe. Inclusion of the chromosome 17 probe in such cases had proved to be robust and reproducible between other laboratories.[64] A correlation of 100% was found between CISH and FISH in one study in which samples scoring more than two signals per nucleus were controlled using a chromosome 17 CISH probe on adjacent tissue.[45] Using this approach, breast cancers with aneusomy or polysomy of chromosome 17 can be distinguished from genuine low-level *HER2* amplification; however, FISH was still deemed useful in some instances.[68] It has been suggested that all breast carcinomas with a *HER2* copy number of 2–7 by FISH should also be analysed for chromosome 17.[69] A similar algorithmic use of a chromosome 17 probe by CISH would require less time and resources than evaluating *HER2* and chromosome 17 in a routine fashion on all cases.

Recently, Gong *et al* published a multicentre study examining the ability to detect *HER2* gene status in breast cancer comparing conventional scoring criteria with the new ASCO/CAP recommendations.[66] The key difference between the ASCO/CAP guidelines and USA FDA approved manufacturer scoring criteria for *HER2* amplification by CISH (Zymed SPOTLight HER2 CISH; Invitrogen, Camarillo, California, USA) and FISH (Vysis PathVysion) is that no equivocal category is used in the manufacturer's scoring criteria. The current ASCO/CAP guidelines for *HER2* detection by FISH have criteria for non-amplified (*HER2*/CEP17 ratio <1.8), equivocal (*HER2*/CEP17 ratio 1.8–2.2), and amplified (*HER2*/CEP17 ratio >2.2). Similarly, the ASCO/CAP guidelines for non-amplified, equivocal and amplified cases detected by CISH are <4, 4–6, and >6, respectively. The authors concluded that the concordance between CISH and FISH for positive and negative cases of *HER2* amplification was excellent using the guidelines of the manufacturer and those of ASCO/CAP. Slightly higher concordance rates and reproducibility were achieved at the two scoring sites using the ASCO/CAP guidelines.

Evaluation of the available published data leads to the conclusion that bright field techniques, such as CISH, have potential to be used as an alternative to FISH testing in the assessment of *HER2* status in breast cancers. In general, CISH is thought to offer several advantages over the FISH technique including: the ability to archive CISH prepared material indefinitely, the use of a conventional bright field microscope to interpret staining, the simultaneous assessment of morphology and gene copy number in the same slide, and the identification of tumour heterogeneity using low-level magnification.[12] In addition, CISH is CE marked and FDA approved. Many of these same advantages are possible through use of another type of bright field in situ hybridisation based on metallographic, rather than chromogenic, probe detection.

Metallographic in situ hybridisation

Unlike CISH, enzyme metallographic in situ hybridisation utilises an enzymatic reaction to facilitate the deposition of metal directly from solution to identify the target site. In addition to the advantages offered by chromogenic bright field in situ hybridisation, metallographic in situ hybridisation provides higher sensitivity and resolution for both amplified and non-amplified genes. An excellent review of metallographic bright field in situ hybridisation modalities was recently published[70]; discussion herein will be focused on describing the principles, practicalities and relative utility of this technology.

Due to limitations of early Nanogold-silver enhancement procedures that made them cumbersome for routine use, a simplified gold-enhanced Nanogold-streptavidin method, termed gold-facilitated in situ hybridisation (GOLDFISH) was developed to assess *HER2* gene status (figure 3).[71] This technique, initially developed as a simplified way to qualitatively identify confluent amplification signals in tissue sections rather than a quantitative assessment of discreet dots, demonstrated much initial promise.[72] The first generation gold-facilitated autometallographic bright field in situ hybridisation displayed good interobserver interpretative reproducibility in an examination of a series of 66 breast carcinomas[73]; however, the need to differentiate cases with chromosome 17 aneusomy or polysomy from those with low-level *HER2* amplification necessitated use of a quantitative interpretation method.[72]

It was subsequently discovered that horseradish peroxidase can be used to selectively deposit metal from solution in the absence of a particulate nucleating agent such as Nanogold. The basic principles of this new technology, EnzMet, are presented in figure 4A. As commercialised enzyme metallography is known as silver in situ hybridisation (SISH). This advancement produced discreet spots of metallic silver deposition, from the enzymatic action of peroxidase on silver acetate in the presence of hydroquinone, at the target site, allowing a superior quantitative assessment of gene copy number.[72] Results of the staining achieved by this method are demonstrated in figure 4B,C. The EnzMet Gene Pro assay, a form of SISH that incorporates concomitant protein detection, has demonstrated excellent interobserver reproducibility,[74 75] and several studies have now

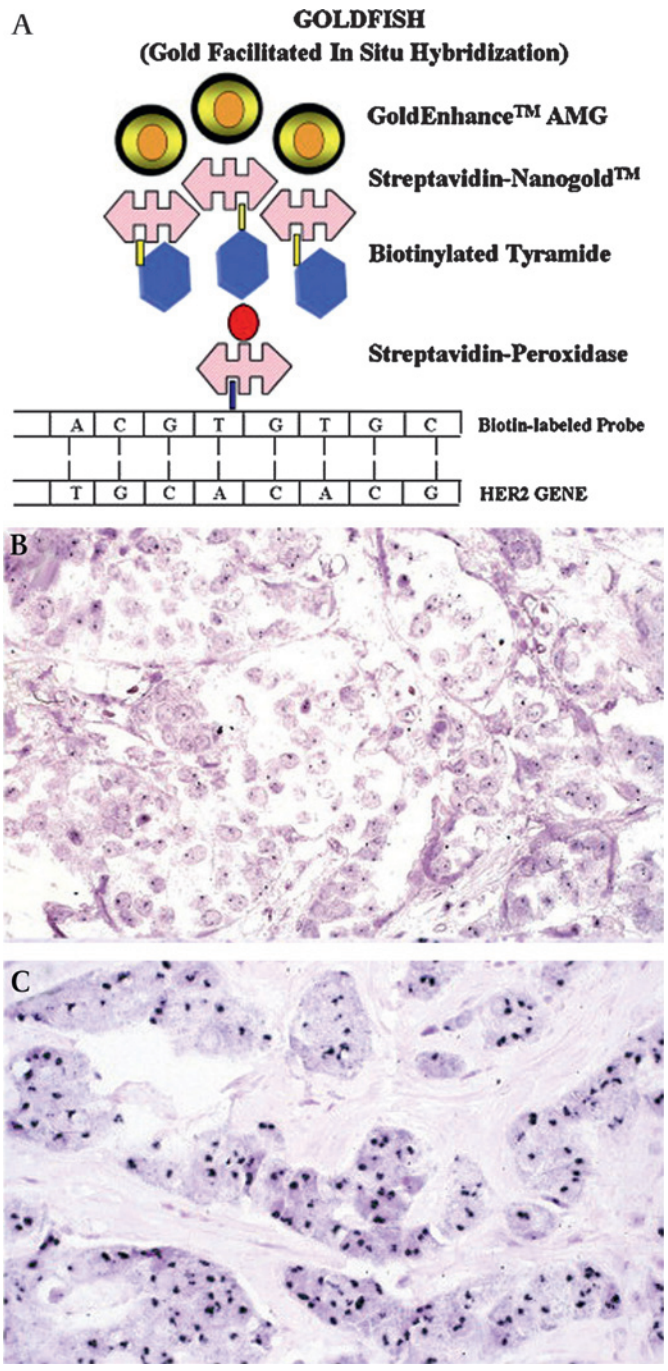

Figure 3 (A) Schematic of gold-facilitated in situ hybridisation (GOLDFISH) assay demonstrating recognition of the biotin-labelled probe with a biotinylated tyramide followed by strepavidin-Nanogold (Nanoprobes). The Nanogold particulate nucleating agent facilitates autometallographic deposition of gold from a solution of silver acetate in the presence of hydroquinone at the target site. (B) Example of *HER2* detection using the GOLDFISH technique with demonstration of non-amplified *HER2* in infiltrating ductal carcinoma producing 1—2 signals per nucleus. (C) Example of GOLDFISH technique in a breast carcinoma containing amplified *HER2* that is demonstrated by multiple large confluent nuclear signals. Original magnification ×400. Reprinted from Powel *et al*[70] with permission from Elsevier.

been published that compare *HER2* gene status in breast carcinomas as determined by SISH and FISH (table 2).

To date, the published studies examining *HER2* gene copy number by SISH have evaluated relatively small series of usually less than 100 breast cancers each. In 2007, Dietel *et al* reviewed a series of 99 invasive breast carcinomas by automated SISH and FISH.[76] The results were analysed using the ASCO/CAP guidelines. Overall concordance was 96%. Discrepant cases were usually attributable to the presence of intratumoral heterogeneity of *HER2* amplification. The authors concluded that SISH was as reliable as FISH in determining *HER2* amplification.

One year later, Carbone *et al* published a multicentre study that examined the staining and interpretative reproducibility of the *HER2* SISH assay (Ventana Medical Systems, Tucson, Arizona, USA) from 89 breast carcinomas using multiple techniques.[63] The reproducibility and efficacy of *HER2* SISH staining was excellent (median K_w value 0.91). Overall concordance between positive and negative SISH and FISH results was also superb (93—100%). However, concordance between SISH and FISH was lower (50%) for a group of eight cases in which the *HER2*/CEP17 ratio was between 1.5 and 3.0. These results suggest that the low-level or intermediate category of amplification poses challenges for the SISH, as well as the CISH, method of testing. This specific question has not been systematically addressed for FISH to our knowledge.

In 2009, Sousha *et al* published an evaluation of *HER2* amplification in 53 breast cancers by automated SISH and FISH.[77] In 94% of the cases examined, SISH and FISH results were identical using scoring criteria provided by the manufacturer. Two of the breast cancers were negative for *HER2* amplification by SISH and positive by FISH. Another breast cancer was scored negative by FISH and positive for amplification by SISH. The authors agreed with the conclusion reached by Dietel *et al* in stating that automated SISH detection of *HER2* in excised breast cancers compares very favourably with FISH analysis.

A recent study examining 230 breast cancers with a rapid SISH scoring technique determined a very high concordance (99.6%) with FISH testing.[78] The authors employed a 'SISH quick-score' when evaluating *HER2* status by SISH. Similar to the FDA-approved assessment of *HER2* status with a single CISH probe, the SISH quick-score relies upon the number of stain dots present in tumour cell nuclei. With dots from an epithelial or fibroblast cell used as a reference signal, the scorers evaluated *HER2* status as: non-amplification, aneusomy, polysomy, and low-level or high-level amplification. The two evaluators in this study were 100% concordant in their interpretation using the SISH quick-score technique. In addition to confirming the ability of automated SISH to accurately assess *HER2* status in breast cancers, the data of the study suggest that the use of SISH quick-score is of additional utility in that it combines the resolution of SISH the with straightforward interpretation style of the CISH scoring method.

In sum, the studies available for review suggest that SISH is a reliable substitution for FISH in the determination of *HER2* status in invasive breast carcinoma. Similar to FISH, SISH allows enumeration of *HER2* and chromosome 17 signals enabling generation of a *HER2*/CEP17 ratio. The published ASCO/CAP guidelines, including the equivocal range of *HER2* gene amplification (*HER2*/CEP17 ratio of 1.8—2.2), are also readily applied to this bright field in situ hybridisation. The benefits of SISH detection of *HER2* include: very high sensitivity with high resolution and signal separation, accurate quantitation of gene amplification, excellent visualisation of tissue morphology, and adaptability for automation.[70] SISH is currently CE marked but has not yet been FDA approved. While assessment of *HER2* using currently available commercial technology opens up the benefits of the CISH and SISH platforms to laboratories not able to perform FISH, additional advances in bright field in situ hybridisation technologies are currently under development.

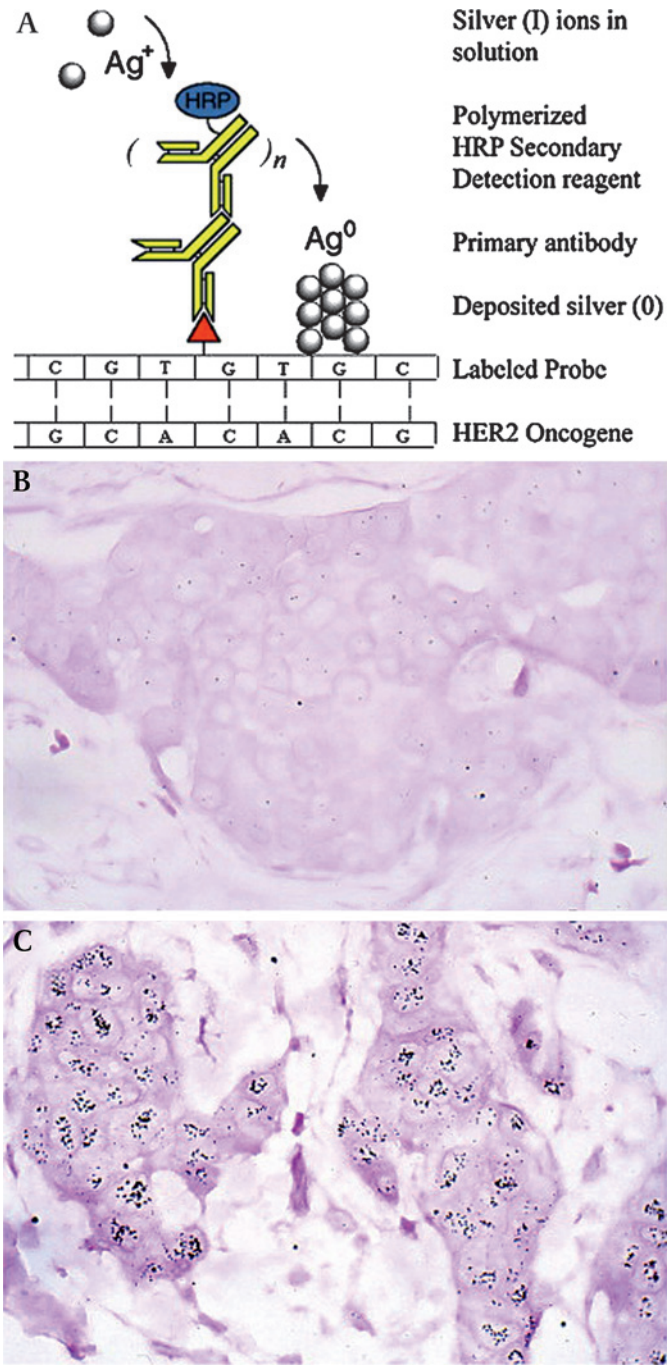

Figure 4 (A) Schematic of enzyme metallography demonstrating detection of the probe with a primary anti-hapten antibody followed by a horseradish peroxidase (HRP)-labelled secondary antibody. Enzyme-catalysed deposition of metallic silver from the silver acetate solution, in the presence of hydroquinone, then occurs at the target site. (B) Example of *HER2* detection using the enzyme metallography EnzMet technique with demonstration of non-amplified breast cancer; 1–2 signals are present in each nucleus. (C) Example of breast carcinoma containing amplified *HER2*; multiple distinct signals are present in each nucleus. Original magnification ×400. Reprinted from Powel *et al*[70] with permission from Elsevier.

THE FUTURE IS BRIGHT

Dual-colour FISH is considered the 'gold standard' for in situ assessment of gene copy number, in part because of the superior spatial resolution offered by this technique and the FDA approval status of Vysis PathVysion.[12] However, dual colour FISH has the same disadvantages as single-colour FISH and the additional limitation that probes producing more intense signal may lead to the interpretation of biased ratios favouring the brighter probe. Despite some limitations, the ability to directly assess both and multiple targets in the same nucleus simultaneously is highly desirable. Although studies evaluating multicolour detection procedures for bright field microscopy using chromosome specific probes had been reported in the past,[80–82] development of dual-colour CISH using probes for *HER2* and chromosome 17 was reported more recently using single-colour detection of a digoxigenin-labelled *HER2* probe and a biotin labelled chromosome 17 probe.[83] The results of dual-coloured CISH and FISH in that study showed high concordance (91%, κ coefficient 0.82), and the contrast provided by the two colours allowed for immediate distinction between *HER2* amplification and chromosome 17 aneuploidy.[84] Additional reports of dual-colour CISH for the assessment of *HER2* gene status found excellent concordance when respectively compared with FISH results (98.6% and 94.6%).[67 85] Additional advancements in bright field in situ hybridisation are aiming to provide assessment of both *HER2* and chromosome 17 through techniques to identify both targets either

Table 2 Comparison of *HER2* status using SISH and FISH methodologies

Reference	Sample size	No. of test sites	Concordance (%)†	κ coefficient*
Sinczak-Kuta[61]	63	1	NS	0.38
Dietel *et al*[76]	99	5	96.0	0.75
Carbone *et al*[63]	89	5	98.9	NS
Shousha *et al*[77]	53	1	94.0	NS
Collins *et al*[78]	230	2	99.6	NS
Bartlett *et al*[79]	45	7	96.0	NS

FISH, fluorescence in situ hybridisation; SISH, silver in situ hybridisation; NS, not specified.
*95% confidence level unless otherwise specified, coefficients rounded to two decimal places.
†Weighted averages were calculated in some instances.

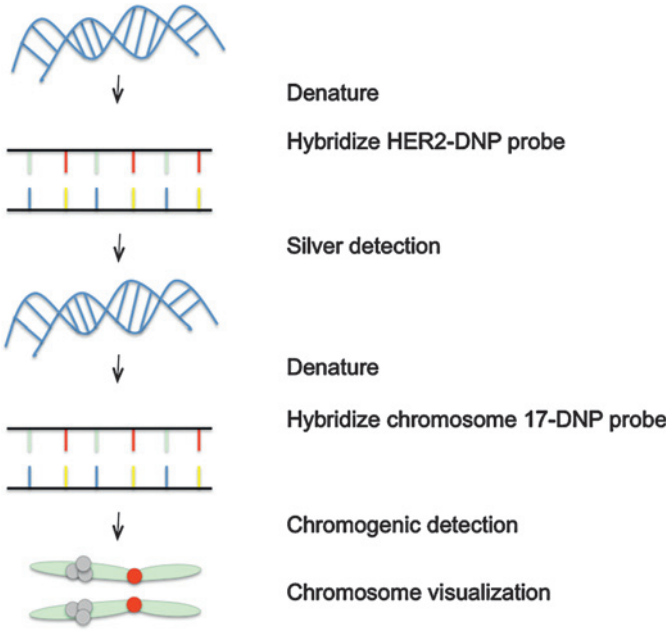

Figure 5 Conceptual schematic demonstrating dual detection of *HER2* and chromosome 17 by bright field double in situ hybridisation. By this technique, dual detection can be accomplished using individual single haptens. The two probes are incompatible and two rounds of target DNA denaturation, hybridisation and stringency washes are carried out sequentially. DNP, dinitrophenol.

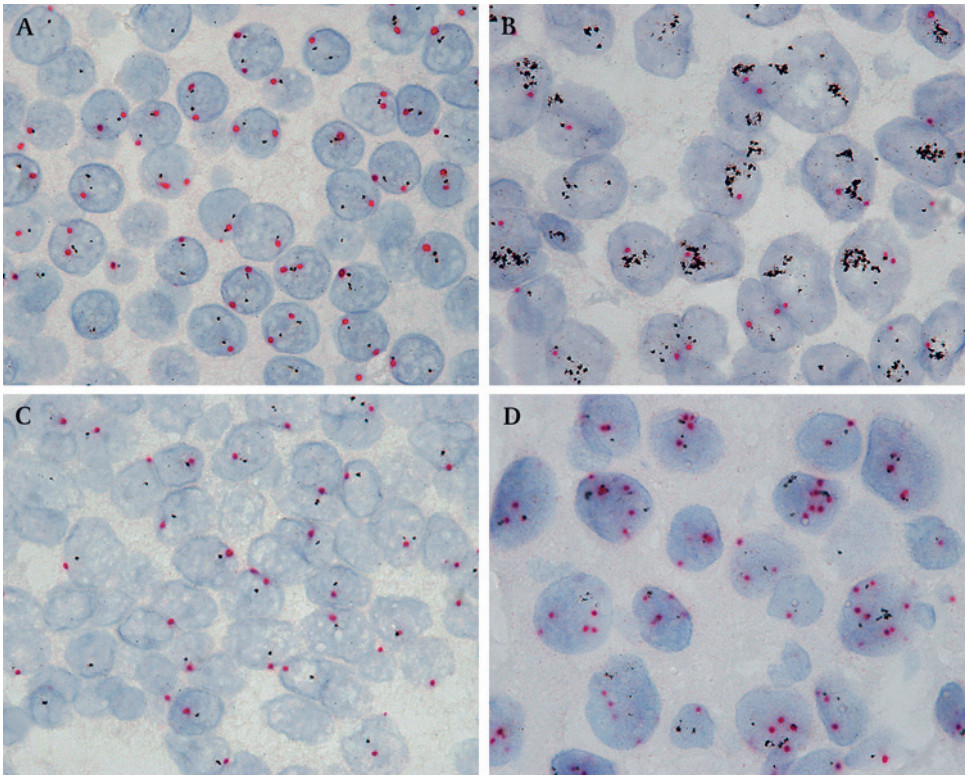

Figure 6 Examples of *HER2* and chromosome 17 detection in non-amplified (A) and amplified (B) breast carcinomas using the dual-colour dual-hapten approach. Single *HER2* gene (C) and chromosome 17 polysomy (D) are demonstrated using bright field double in situ hybridisation. Magnification ×100. Reproduced from Nitta *et al*[86] with author permission.

simultaneously or consecutively (figure 5). Examples of the staining produced by such techniques are demonstrated in figure 6.

Recently, automated bright field double in situ hybridisation (BDISH) applications have been described.[86] In the study by Nitta *et al*, high consensus concordance was demonstrated between FISH and BDISH methods. Future versions of this approach will use simultaneous hybridisations with dual haptens allowing dual colour detection (DISH) of *HER2* and chromosome 17. Depending on the scoring criteria used (historical versus ASCO/CAP) and whether FISH equivocal cases were included, the concordance percentages ranged between 95.7% and 100% (κ coefficients 0.89–1.0). Since publication of that study, the utility of this technology has become apparent,[87] and it has been suggested that the BDISH automated technique might be used in replacement of manual dual-colour FISH methods in the future.[86] Alternatively, techniques combining IHC and BDISH methods may become the new preferred method of *HER2* assessment.[87]

In conclusion, the constant elucidation of the molecular pathogenesis of disease requires that detailed genetic information guide clinical decision making and therapeutic strategies. This demand has placed pressure upon clinical laboratories to provide testing platforms capable of accurately assessing genomic signatures. In situ hybridisation techniques are, and should continue to be, an important part of the pathologist's role in the movement towards personalised medicine.[88] The bright field in situ hybridisation techniques presented offer a glimpse at where the state of diagnostics and pharmacogenomic testing is headed in terms of accurately assessing the morphological status and molecular status of a tumour cell simultaneously.

Take-home messages

- Bright field in situ hybridisation is a molecular technique that enables visualisation of cellular target DNA using chromogenic (eg, chromogenic in situ hybridisation) or enzyme metallographic (eg, silver in situ hybridisation) methods of detection with conventional light microscopy.
- Benefits to bright field in situ hybridisation include: the ability to archive prepared material indefinitely, the use of a conventional bright field microscope to interpret staining, the simultaneous assessment of morphology and gene copy number on the same slide, and the identification of tumour heterogeneity using low-level magnification.
- An informal analysis of the literature demonstrates excellent concordance among published comparisons of bright field in situ hybridisation and fluorescence in situ hybridisation assessment of *ERBB2* (*HER2*) gene status in breast carcinoma.
- Current American Society for Clinical Oncology and College of American Pathologists interpretation guidelines for FISH detection of *ERBB2* (*HER2*) gene status in breast carcinoma are readily applied to bright field in situ hybridisation techniques.
- Effective advocacy, use and interpretation of bright field in situ hybridisation can be an important part of the role of the pathologist in the movement towards personalised medicine.

Interactive multiple choice questions

This JCP review has an accompanying set of multiple choice questions (MCQs). To access the questions, click on BMJ Learning: Take this module on BMJ Learning from the content box at the top right and bottom left of the online article. For more information please go to: http://jcp.bmj.com/education Please note: the MCQs are hosted on BMJ Learning—the best available learning website for medical professionals from the BMJ Group. If prompted, subscribers must sign into JCP with their journal's username and password. All users must also complete a one-time registration on BMJ Learning and subsequently log in (with a BMJ Learning username and password) on every visit.

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