Fluorescence (FISH) and chromogenic (CISH) *in situ* hybridisation in prostate carcinoma cell lines: comparison and use of virtual microscopy

K. ELLIOTT[*], P. W. HAMILTON[†] and P. MAXWELL[*,†]
[*]*Department of Pathology, Belfast Health & Social Care Trust, and*
[†]*Centre for Cancer Research and Cell Biology, Queen's University, Belfast*

Accepted: 10 July 2008

ABSTRACT

Chromogenic *in situ* hybridisation (CISH) has become an attractive alternative to fluorescence *in situ* hybridisation (FISH) due to its permanent stain which is more familiar to pathologists and because it can be viewed using light microscopy. The aim of the present study is to examine reproducibility in the assessment of abnormal chromosome number by CISH in comparison to FISH. Using three prostate cell lines – PNT1A (derived from normal epithelium), LNCAP and DU145 (derived from prostatic carcinoma), chromosomes 7 and 8 were counted in 40 nuclei in FISH preparations (x100 oil immersion) and 100 nuclei in CISH preparations (x40) by two independent observers. The CISH slides were examined using standard light microscopy and virtual microscopy. Reproducibility was examined using paired Student's *t*-test ($P<0.05$). Reproducibility between observers was good for both FISH and CISH. No significant differences in chromosome count were seen between the techniques. Chromosomes 7 and 8 showed disomic status for each cell line except LNCAP, which proved to be heterogeneous (disomic/aneusomic), particularly for chromosome 8. Virtual microscopy proved to be easy to use and gave no significant differences from standard light microscopy. These results support the hypothesis that there is no significant difference between FISH and CISH techniques.

KEY WORDS: Chromosomes, human, pair 7.
Chromosomes, human, pair 8.
In situ hybridization, chromogenic.
In situ hybridization, fluorescence.
Prostatic neoplasms.

Introduction

Molecular pathology has revolutionised our understanding of DNA and the role it plays in the disease process. Molecular techniques remain a main focus of research, with *in situ* hybridisation (ISH) being a popular technique. This is currently evolving from an impermanent fluorescent-labelled method (FISH), which has become established for example in breast cancer, to a more permanent and accessible preparation using a chromogenic stain (CISH), particularly useful in gene amplification in breast cancer.[1] However, this transition from fluorescence to chromogenic methodology requires further research to confirm its reproducibility and specificity.[2–6]

A new area of investigation is the application of CISH to the determination of abnormal chromosome number in prostate carcinoma, which is the most common cause of cancer death in men in the Western world.[7] Chromosomes 7 and 8 have been shown to be particularly important in prostate cancer, with several studies showing significant aneusomy for both chromosomes and a relationship to Gleason scores T3 and T4.[8–10]

The other advantage of CISH is that it is more amenable to the application of virtual microscopy (VM), where the entire tissue sample can be scanned at high resolution for digital archiving, viewing across the internet, online scoring and computerised image analysis.[11–15] While virtual fluorescence microscopy is available, most commercial systems only permit scanning based on brightfield microscopy. By combining CISH and VM, new opportunities allow sequence-based biomarkers to be evaluated on whole-slide images through online sharing of slides, web-based scoring systems and by quantitative image analysis. However, this is a relatively new concept in histopathology and specifically in CISH evaluation, and therefore requires further research.

The aim of the present study is to determine the chromosomal status of chromosomes 7 and 8 in prostate cancer cell lines using FISH and CISH on paraffin-wax embedded material and to assess chromosomal copy number assessment by manual and virtual microscopy.

Correspondence to Dr. Perry Maxwell
CCRCB, 97 Lisburn Road, Belfast BT9 7BL
Email: p.maxwell@qub.ac.uk

Materials and methods

Prostate cell lines PNT1A, LNCAP (European Cell Collection, UK) and DU145 (ATCC-LGC, UK) were purchased, grown to confluence, passaged and harvested from appropriate growth media. Cell pellets were fixed in 10% formalin for 24 h, mixed with 1% agarose and the resulting pellet processed to paraffin wax. Sections (3 μm) from each cell line were used for each experiment.

For the FISH technique, slides were deparaffinised in xylene for a minimum of 10 min, passed through 100% and 95% alcohol to water, and then washed in buffer (Dako, UK)

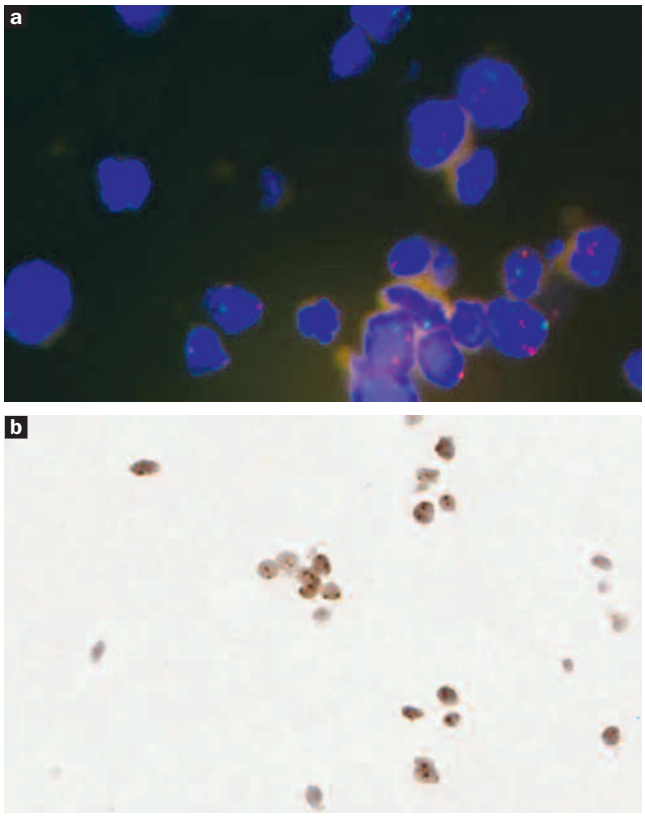

Fig. 1. Staining of PNT1A cell line: a) FISH (chromosome 7 [green], chromosome 8 [red], original magnification x1000) and b) CISH (original magnification x400).
See these images in colour at www.bjbs-online.org

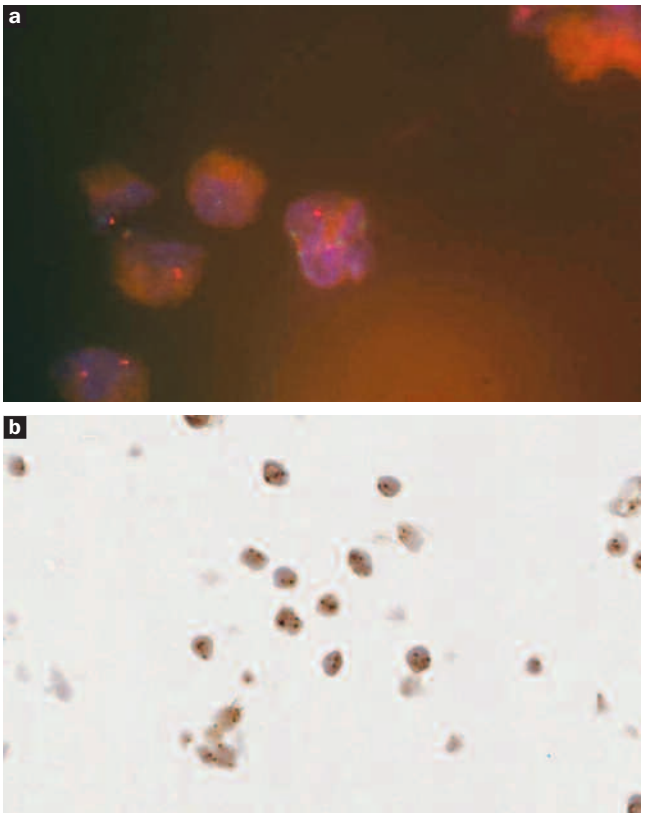

Fig. 2. Staining of DU145 cell line: a) FISH (chromosome 7 [green], chromosome 8 [red], original magnification x1000) and b) CISH (original magnification x400).
See these images in colour at www.bjbs-online.org

for 2 min. Pretreatment solution (sodium thiocyanate, Dako) was sealed onto the slide using Dako sealant and a coverslip (22 x 22 mm), incubated for 10 min at 98°C and then placed on a hybridising unit (Dako) at 37°C for 15 min. The slides were then washed in buffer (Dako) for 2 min (x3), digested with pepsin (Dako) for 15 min at room temperature then washed (x3) for 2 min in buffer prior to dehydration in 100% alcohol for 5 min (x2). Slides were then air-dried and 10 µL of the probe (chromosome 7: PlatinumBright 495-labelled; chromosome 8: PlatinumBright 550-labelled, Kreatech, The Netherlands) was applied to the slide and sealed. The probe and cells were denatured at 75°C for 10 min and then hybridised at 37°C for 20 h. Unbound probe was removed in 0.05 SSC at 63°C for 5 min in a water bath. Nuclei were stained with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI)-containing mounting medium (Vector, Peterborough, UK) and coverslipped.

Fluorescence signals were counted in 40 nuclei (non-overlapping cells) by two independent observers (KE, PM) using a Leica CTR6000 fluorescence microscope under green, red and blue filters.

For the CISH technique, sections were deparaffinised and passed through 100% and 95% alcohol to distilled water. Pretreatment solution (sodium thiocyanate, Zymed, UK) was sealed onto the slide using Dako sealant and a coverslip (22 x 22 mm), incubated for 10 min at 98°C then placed on the hybridiser at 37°C for 15 min. Further washing (x3) of the slides in wash buffer (Dako) for 2 min each was followed by enzyme digestion (Zymed) for 15 min at room temperature. Washing (x3) for 2 min in distilled water was followed by dehydration in 100% alcohol (x2) for 5 min and air-drying. Then, 10 µL of biotin-labelled probe (chromosome 7, Zymed) was sealed on the slide. Both probe and cells were denatured at 95°C for 5 min and hybridised at 37°C for 20 h. Slides were then placed in Zymed stringency wash at 75°C for 5 min in a water bath, and then washed (x3) in Tris-buffered saline for 2 min. The chromosome 7 probe was detected using mouse anti-biotin (1 in 80 dilution, Dako) for

Table 1. Intra-observer and inter-observer reproducibility of assessing chromosomes 7 and 8 in each of the cell lines by the FISH technique. Values are derived from two observations of 100 nuclei per sample. No significant difference between observers was seen ($P<0.05$).

	PNT1A				DU145				LNCAP			
	Ch7		Ch8		Ch7		Ch8		Ch7		Ch8	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Observer 1	75	76	78	79	86	96	89	93	98	79	9	84
Observer 2	73	75	76	76	86	84	87	88	91	91	79	98

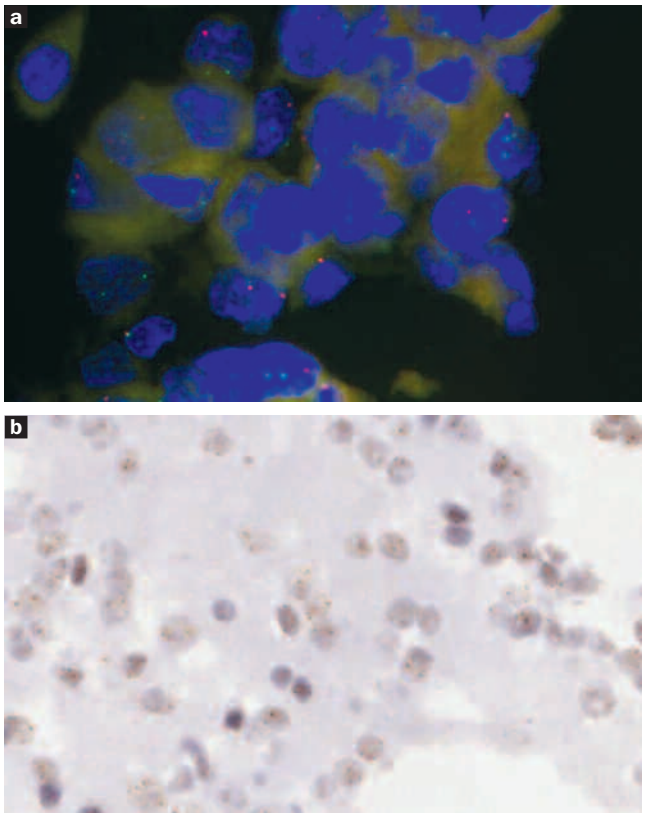

Fig. 3. Staining of LNCAP cell line: a) FISH (chromosome 7 [green], chromosome 8 [red], original magnification x1000) and b) CISH (original magnification x400).
See these images in colour at www.bjbs-online.org

30 min. Application of polymer EnVision (Dako) peroxidise anti-mouse for 25 min was followed by DAB for approximately 1 min and then a washed in distilled water. Cell lines were lightly counterstained in Harris' haematoxylin.

Two independent observers (KE, PM) counted 100 nuclei (non-overlapping cells). This was repeated for reproducibility statistics. All fields were observed under a light microscope using a x40 objective.

In order to assess the performance of virtual microscopy, CISH cell lines were scanned using an Aperio T3 scanner (Aperio, CA) using a x40 objective and then uploaded to the internet server. The images were viewed on a computer with access to the internet and viewed at the equivalent of x400 magnification. Two independent observers (KE, PM) counted 100 nuclei (non-overlapping cells) in each section. This was repeated for reproducibility statistics.

Training sets using one section from each cell line were prepared and all staining protocols and assessment criteria were established.

For each set of results, statistical analysis included sum, mean, standard deviation and mode of observed counts. To test intra-observer and inter-observer reproducibility, a paired Student's *t*-test (two-tailed) was calculated. Intra-observer and inter-observer reproducibility was calculated for all results.

Results

Figures 1, 2 and 3 show the outcomes of both FISH and CISH staining for all cell lines.

Reproducibility in assessing chromosome status by each of the observers was good. There was no significant difference between the scores for each observer (intra-observer reproducibility, paired Student's *t*-test $P<0.05$) except with the LNCAP cell line when using the FISH technique. This may indicate chromosome number heterogeneity and was confirmed in the frequency distribution shown in Table 1. Inter-observer reproducibility for the CISH method (assessed using either standard light or virtual microscopy) was good and no significant difference was noted between the observers (Table 2, $P<0.05$). There was no significant difference between CISH and FISH counts for chromosome 7.

There was good agreement in the trends in frequency distribution between FISH and CISH for chromosome number, from the 'normal' PNT1A cell line through the malignant DU145 and LNCAP cell lines (Figs. 4 and 5).

Figure 4 illustrates the frequency distribution of chromosomes 7 and 8 using FISH. The PNT1A cell line showed that most of the nuclei were disomic for chromosomes 7 and 8, although there were some aneusomic cells present. The LNCAP and DU145 cell lines showed disomy for chromosome 7, with an increase in aneusomic cells compared to PNT1A. However, the LNCAP cell line showed a greater increase in aneusomy for chromosome 8.

Figure 5 illustrates the frequency distribution of chromosome 7 using CISH, comparing light microscopy and virtual microscopy. The PNT1A cell line showed mostly disomic cells and a few aneusomic cells, and the LNCAP and DU145 cell lines showed chromosome 7 disomy and increased aneusomy compared with the PNT1A cell line.

The virtual microscopy images stored on the server proved easy to access. It was possible to view the section in its entirety then move to the magnification at which it was scanned. Also, it was possible to incorporate a small screen for navigation around the slide, which aided unbiased, random sampling. Furthermore, both observers could access the images on demand.

Discussion

Currently, FISH is the gold standard technique for HER-2:chromosome 17 ratio assessment.[16] However, CISH

Table 2. Inter-observer reproducibility of assessing chromosome 7 by CISH in each of the cell lines by standard light and virtual microscopy. Values are derived from two observations of 100 nuclei per sample ($P<0.05$).

	PNT1A		DU145		LNCAP	
	OB1	OB2	OB1	OB2	OB1	OB2
Standard light microscopy	194	193	231	222	239	227
Virtual microscopy	173	192	215	222	211	241

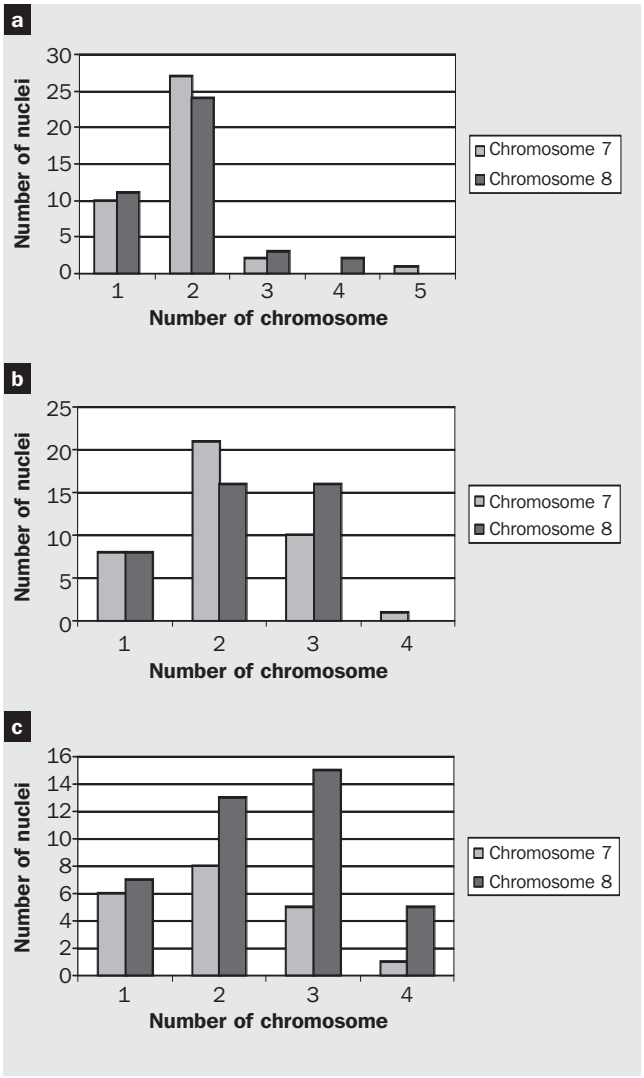

Fig. 4. Frequency distribution of chromosome counts by FISH in a) PNT1A, b) DU145 and c) LNCAP cell lines.

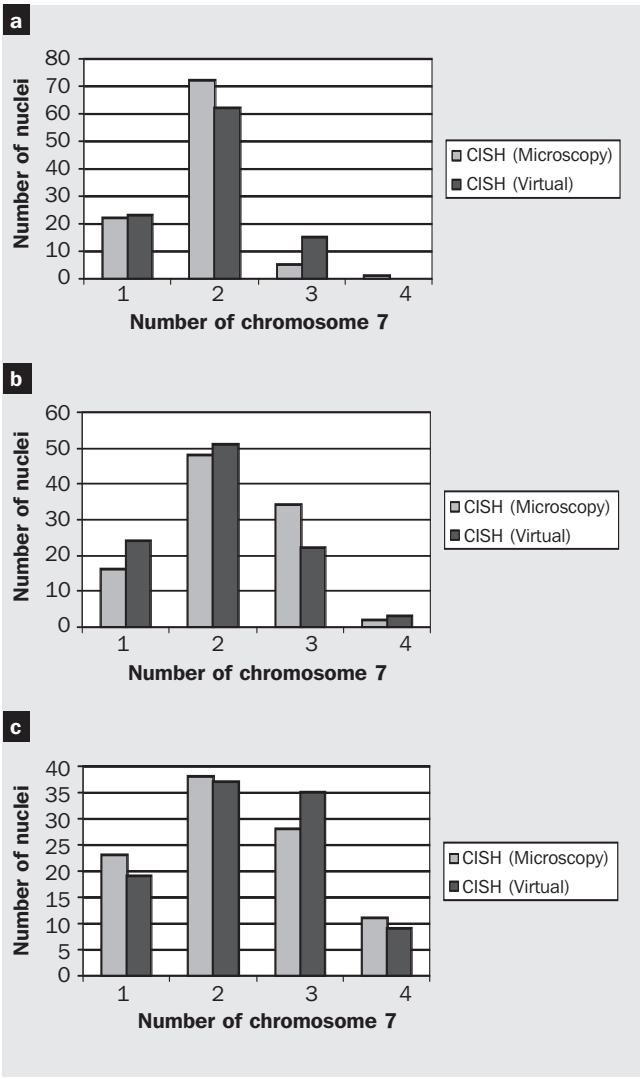

Fig. 5. Frequency distribution of chromosome 7 counts by CISH for standard light microscopy and virtual microscopy in a) PNT1A, b) DU145 and c) LNCAP cell lines.

is a similar technique that may prove to be equally suitable.

The present study found no significant difference in results between FISH and CISH techniques. A training set for each method was used initially to ensure both observers were familiar with visualisation of the staining patterns and agreed scoring criteria. Although there was an indication of some differences within the counts, these were not significant, which indicated good inter-observer reproducibility for FISH and CISH.

Using paired Student's *t*-test ($P<0.05$), no significant difference between FISH and CISH was seen in any of the cell lines investigated. Previous reports have shown concordance between FISH and CISH, where results ranged from 91% to 100%, but these looked at gene amplification rather than chromosome copy number.[2-6]

Optimisation of pretreatment is needed, especially when using fixed and processed material due to the different procedures used. This is particularly important for pepsin digestion time and stringency temperature. In the CISH technique, localisation procedures using the secondary anti-biotin dilution needed optimisation in order to ensure minimal background staining. The training sets used for the observers helped to avoid differences in scoring criteria.

Another source of possible error may be the use of tissue sections where nuclear truncation can complicate evaluation and interpretation.[1,17] However, this can be overcome by the use of nuclear suspensions, which involves isolating whole cell nuclei.[17,18] Stock *et al.* found this to be superior when compared to conventional FISH.[19] In the present study, tissue section reproducibility was maintained by calculating the wandering mean for the determination of the optimum number of nuclei to be assessed as part of the training set.[20] This step seems to be a crucial part in determining chromosome count.

The main drawback to the use of CISH is the lack of dual probing. This may prove to be a problem when two targets need to be examined.[3] Recent commercial developments of dual-labelled probes for CISH may prove useful, but they require further investigation.

Virtual microscopy and the counting of CISH-labelled chromosomes from a computer screen proved to be as reproducible as the standard light microscopy method. Virtual microscopy is an emerging tool in pathology that produces an entire high-resolution digital record of the

microscopic slide and facilitates interactive and automated measurement of tissue and cellular markers both locally and remotely across the internet.[12,13,15] This makes quantitative evaluation of a microscopic image more acceptable. It does not rely on taking numerous digital photographs of varying quality from a single sample, but all measurements can be derived from one virtual slide image. Several commercial companies now market virtual slide scanners, making this approach amenable to most laboratories for archiving and analysis of tissue-based research samples. In the present study, counting CISH-labelled chromosomes using virtual microscopy proved to be easy and straightforward, and the image quality was excellent.

Virtual microscopy is no longer restricted to light microscopy. Several companies now produce systems capable of generating virtual slides under fluorescent conditions, which provides the opportunity to undertake virtual slide-based measurement using FISH.

During assessment of FISH-stained nuclei in the PNT1A cell line, it was apparent that most nuclei were diploid for chromosomes 7 and 8; however, some nuclei were aneusomic. This finding agrees with the results of the study by Beheshti *et al*.[21] A possible reason for this aneusomic population is genetic drift through instability brought about via immortalisation of the cell line. The prostate carcinoma-derived cell line LNCAP showed heterogeneity for chromosome 8. The other cell lines examined by FISH and CISH appeared to be disomic. These results indicate that the use of the PNT1A, DU145 and LNCAP cell lines may be a useful model for investigating prostatic carcinoma, in which the aneusomic status of chromosomes 7 and 8 has been described.[8–10]

In conclusion, the results show that CISH is as reproducible as FISH and can be used as an alternative, unless dual probes are required. Virtual microscopy proved to be as sensitive as light microscopy and may be used as an alternative. It is useful when two observers are counting the slides. Therefore, use of virtual microscopy and CISH in the assessment of chromosomal number in experimental studies on cell lines and tissue sections is recommended. □

References

1 Lambros MB, Natrjan R, Reis-Filho J. Chromogenic and fluorescent *in situ* hybridization in breast cancer. *Hum Pathol* 2007; **38**: 1105–22.

2 Gong Y, Gilcrease M, Sneige N. Reliability of chromogenic *in situ* hybridization for detecting *HER-2* gene status in breast cancer: comparison with fluorescence *in situ* hybridization and assessment of interobserver reproducibility. *Mod Pathol* 2005; **18**: 1015–21.

3 Isola J, Tanner M, Forsyth A, Cooke TG, Watters AD, Bartlett JM. Interlaboratory comparison of *HER-2* oncogene amplification as detected by chromogenic and fluorescence *in situ* hybridization. *Clin Cancer Res* 2004; **10**: 4793–8.

4 Vocaturo A, Novelli F, Benevolo M *et al*. Chromogenic *in situ* hybridization to detect *HER-2/neu* gene amplification in histological and ThinPrep-processed breast cancer fine-needle aspirates: a sensitive and practical method in the trastuzumab era. *Oncologist* 2006; **11**: 878–86.

5 Di Palma S, Collins N, Faulkes C *et al*. Chromogenic *in situ* hybridization (CISH) should be an accepted method in the routine diagnostic evaluation of HER2 stains in breast cancer. *J Clin Pathol* 2007; **60**: 1067–8.

6 Tanner M, Gancberg D, Di Leo BA *et al*. Chromogenic *in situ* hybridization. A practical alternative to fluorescence *in situ* hybridization to detect *HER-2/neu* oncogene amplification in archival breast cancer samples. *Am J Pathol* 2000; **157** (5): 1467–72.

7 Hughes S, Yoshimoto M, Beheshti B, Houlston RS, Squire JA, Evans A. The use of whole genome amplification to study chromosomal changes in prostate cancer: insights into genome-wide signature of preneoplasia associate with cancer progression. *BMC Genomics* 2006; **7**: 65.

8 Gallucci M, Merola R, Farsetti A *et al*. Cytogenetic profiles as additional markers to pathological features in clinically localized prostate carcinoma. *Cancer Lett* 2006; **237**: 76–82.

9 Skacel M, Ormsby AH, Pettay JD *et al*. Aneusomy of chromosomes 7, 8 and 17 and amplification of *HER-2/neu* and epidermal growth factor receptor in Gleason score 7 prostate carcinoma: a differential fluorescent *in situ* hybridization study of Gleason pattern 3 and 4 using tissue microarray. *Hum Pathol* 2001; **32** (12): 1392–7.

10 Alcaraz A, Corral JM, Ribal MJ *et al*. Fluorescence *in situ* hybridization analysis of matched primary tumour and lymph node metastasis of D1(pT2-3pNIMO) prostate cancer. *BJU Int* 2004; **94**: 407–11.

11 Goldberg HR, Dintzis R. The positive impact of team-based virtual microscopy on student learning in physiology and histology. *Adv Physiol Educ* 2007; **31**: 261–5.

12 Lundin M, Lundin J, Helin H, Isola J. A digital atlas of breast histopathology: an application of web-based virtual microscopy. *J Clin Pathol* 2004; **57**: 1288–91.

13 Mikula S, Trotts IS, Stone JM, Jones EG. Internet-enabled high-resolution brain mapping and virtual microscopy. *Neuroimage* 2007; **35**: 9–15.

14 Romer DJ, Yearsley KH, Ayers LW. Using a modified standard microscope to generate virtual slides. *Anat Rec B New Anat* 2003; **272** (1): 91–7.

15 Stewert J 3rd, Miyazaki K, Bevans-Wilkins K, Ye C, Kurtycz DF, Selvaggi SM. Virtual microscopy for cytology proficiency testing. Are we there yet? *Cancer Cytopathol* 2007; **111**: 203–9.

16 Kammori M, Kurabayashi R, Kashio M *et al*. Prognostic utility of fluorescence *in situ* hybridization for determining *HER2* gene amplification in breast cancer. *Oncol Rep* 2008; **19**: 651–6.

17 Gelpi E, Ambros IM, Birner P *et al*. Fluorescent *in situ* hybridization on isolated tumour cell nuclei: a sensitive method for 1p and 19q deletion analysis in paraffin-embedded oligodendroglial tumour specimens. *Mod Pathol* 2003; **16**: 708–15.

18 Bosga-Bouwer AG, van Imholf GW, Boonstra R *et al*. Follicular lymphoma grade 3B includes three cytogenetically defined subgroups with primary t(14;18), 3q27, or other translocations: t(14;18) and 3q27 are mutually exclusive. *Blood* 2003; **101**: 1149–54.

19 Stock C, Ambros JM, Lion T *et al*. Detection of numerical and structural chromosome abnormalities in paediatric germ cell tumours by means of interphase cytogenetics. *Genes Chromosomes Cancer* 1994; **11** (1): 40–50.

20 Hall PA. Assessing apoptosis: a critical survey. *Endocr Relat Cancer* 1999; **6**: 3–8.

21 Beheshti B, Karaskova J, Park PC, Squire J, Beatty BG. Identification of a high frequency of chromosomal rearrangements in the centromeric regions of prostate cancer cell lines by sequential Giemsa-banding and spectral karyotyping. *Mol Diagn* 2000; **5**: 23–32.