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Automated quantification of FISH signals in urinary cells enables the assessment of chromosomal aberration patterns characteristic for bladder cancer

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ABSTRACT

Targeting the centromeres of chromosomes 3, 7, 17 (CEP3, 7, 17) and the 9p21-locus (LSI9p21) for diagnosing bladder cancer (BC) is time- and cost-intensive and requires a manual investigation of the sample by a well-trained investigator thus overall limiting its use in clinical diagnostics and large-scaled epidemiological studies. Here we introduce a new computer-assisted FISH spot analysis tool enabling an automated, objective and quantitative assessment of FISH patterns in the urinary sediment. Utilizing a controllable microscope workstation, the microscope software Scan^R was programmed to allow automatic batch-scanning of up to 32 samples and identifying quadruple FISH signals in DAPI-scanned nuclei of urinary sediments. The assay allowed a time- and cost-efficient, automated and objective assessment of CEP3, 7 and 17 FISH signals and facilitated the quantification of nuclei harboring specific FISH patterns in all cells of the urinary sediment. To explore the diagnostic capability of the developed tool, we analyzed the abundance of 51 different FISH patterns in a pilot set of urine specimens from 14 patients with BC and 21 population controls (PC). Herein, the results of the fully automated approach yielded a high degree of conformity when compared to those obtained by an expert-guided re-evaluation of archived scans. The best cancer-identifying pattern was characterized by a concurrent gain of CEP3, 7 and 17. Overall, our automated analysis refines current FISH protocols and encourages its use to establish reliable diagnostic cutoffs in future large-scale studies with well-characterized specimens-collectives.

1. Introduction

Bladder cancer (BC) ranks among the ten most common cancers in the world and requires frequent follow-up investigations [1]. Urine cytology and cystoscopy are considered the gold standards for BC detection. Cystoscopy, however, is invasive and bears the risk of misdiagnosing a tumor *in situ* for urocystitis, while non-invasive cytology has limited sensitivity, especially for low-stage-tumors [2]. Numerous approaches have been developed to find non-invasive biomarkers for BC in urine at different molecular levels, and although many outperformed cytology with respect to sensitivity, none achieved its high specificity [3,4]. The BC genome is known to be altered, and studies have assessed the value of chromosomal aberrations to detect BC in exfoliated urothelial cells [5,6]. Previously, a fluorescence-in-situ-hybridization (FISH) test targeting the centromeres of chromosomes 3, 7, 17 and the 9p21-locus has been developed by comparing ten chromosomal targets for the detection of urothelial cancer [7]. The so-called UroVysion™ (Abbott Molecular) was reported to be superior to cytology with improved sensitivity and comparable specificity [2,8]. However, the test exhibits weaknesses which impede its broad application in the urologic community: First,

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processing of urinary FISH samples is time- and cost-intensive [7,9–11]. Moreover, as cells of suspicious morphology are preferentially investigated, the diagnostic value of the UroVysion™ is greatly impaired by its considerable subjectivity regarding specimen-evaluation and the dependency on well-trained investigators [12,13]. Finally, several authors have questioned the evaluation-criteria advised by the Abbott manual and have introduced their own criteria for the identification of BC [2,14–17]. These issues challenged us to develop a computer-assisted high-throughput method by generating reproducible FISH results that can be electronically stored. In addition, the test should enable an objective thus observer-independent (re)assessment of FISH patterns indicative of BC by quantifying any kind of desired FISH signal combinations in all cells of the urinary sediment.

2. Materials and methods

2.1. Study subjects

To test whether our automated FISH assay identifies BC cases, we examined urine samples from cancer patients and controls in a pilot study. Urine specimens from 14 individuals with pathologically confirmed BC (4 females, 10 males, mean age 68 ± 13 years) were obtained from the Department of Urology (Marienhospital Herne, Germany). Four BC patients were diagnosed with non-invasive tumors (pTa), whereas the remaining 10 patients suffered from invasive tumors ($\geqslant$pT1). Urine specimens of 21 population controls (PC) (18 females, 3 males, mean age 67 ± 7 years) were obtained from the Heinz-Nixdorf-RECALL study, a prospective cohort study investigating risk factors for cardiovascular diseases [18]. The study was approved by the Ethics Committee of the Ruhr-University Bochum (No. 3674-10) and the University of Duisburg-Essen (No. 11-4678) and all participants provided written informed consent.

2.2. Collection of the urinary sediment

Urine samples were processed within 1 h as follows: Urine was centrifuged (1000*g*, 10 min) and the pellet was washed in 10 mL PBS. After a second centrifugation (1000*g*, 10 min), the pellet was resuspended in 1 mL fresh glacial acetic acid (3:1) and stored at −20 °C for $\geqslant$30 min. Finally, cells were spun down (1000*g*, 10 min) and resuspended in 200–3000 μL fresh glacial acetic acid depending on the size of the cell pellet.

2.3. Loading of microscope slides with urine sediment

By using an in-house positioning device, we punched 8 holes (3 mm Ø) into a commercially available stick pad. Stuck to a slide, this novel device allowed the placement of up to eight specimens onto a singular slide and at reproducible positions defined by the mold (Fig. 1). By using a commercially available slide holder which can accommodate four slides, our device enabled batch-scanning of 8 × 4 = 32 urine specimens in a single run. To achieve an appropriate density of cells, wells were loaded with 3 × 3 μL aliquots in a stepwise manner. Prior to FISH staining, samples were air-dried for 24 h (RT).

2.4. FISH staining of urine sediment

Cells were prepared for FISH staining using the following treatments: Fixation in 100% methanol (10 min), incubation in 2×SSC, pH 7.0 at 73 °C (2 min), permeabilization with a pepsin solution (ZytoVision GmbH, Bremerhaven, Germany, 10 min), washing in PBS (5 min), incubation in 1% formaldehyde (5 min), washing in PBS (5 min) and dehydration in 70%, 80% and 100% ethanol (1 min each). After the slides had dried, 0.5 μL of a FISH probe mixture targeting centromeres of chromosomes 3 (CEP3, label: spectrum red), 7 (CEP7, label: spectrum green), 17 (CEP17, label: spectrum aqua) and the 9p21 locus (LSI9p21, label: ZyGold) (ZytoLight SPECp16/CEN3/CEN7/CEN17 ZytoVision GmbH, Bremerhaven, Germany) was applied to each well. Wells were individually covered with circular glass cover slips (6 mm Ø, Gerhard Menzel GmbH, Braunschweig, Germany) and sealed with rubber cement. Denaturation (73 °C, 2 min) and hybridization (37 °C, 18 h) were performed in the ThermoBrite Stat Spin® (Abbott Molecular, Des Plaines, IL, USA). After careful removal of rubber and cover glasses, slides were washed twice in 0.4×SSC, 0.3% Nonidet P-40 (pH 7.5, 73 °C, each wash 2 min). Slides were then washed in 2×SSC, 0.1% Nonidet P-40 (pH 7.0, 2 min) followed by immersion in ascending alcohol concentrations (70%, 90%, and 100% ethanol, 1 min each). Nuclei were stained with DAPI (4,6-diamidin-2-phenylindol, AppliChem GmbH, Darmstadt, Germany; 60 ng/μL, 3 min). After rinsing three times in ddH_2O (3 min each), slides were mounted with Dako Fluorescence Mounting Medium (Dako Denmark A/S, Glostrup, Denmark), covered with glass cover slides and stored at 4 °C in the dark until scanning.

2.5. Image acquisition

Slides were scanned using an automated microscope workstation (IX81-ZDC, Olympus, Hamburg, Germany) controlled by an

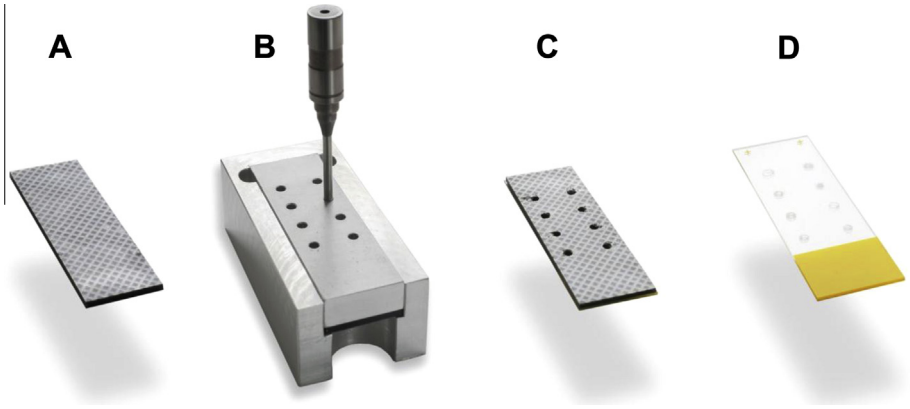

Fig. 1. Newly developed device for the application of FISH specimens. A metal mold enables the punching of circular holes into a pre-cut, commercially available stick pad (A) at reproducible positions using a punch-borer (3 mm Ø) (B). When (reversibly) attached to a slide, the holes serve as wells for specimen-application (C). Specimen-spots on a slide after removal of the stick-pad (D).

IX2-UCB U-HRST2 unit (Olympus, Hamburg, Germany) and equipped with a 100 W Xenon lamp (MT20 burner, Olympus, Hamburg, Germany), using excitation and emission filters for DAPI, FITC, Spectrum red, Spectrum aqua and ZyGold (Suppl. Table S1), a motorized 2-slide x,y,z scanning stage and its control unit (STC, Olympus, Hamburg, Germany), and a digital CCD-camera (C8484-03G02, Hamamatsu Photonics K.K., Japan). The station was driven by the Scan^R version 2.3.0.7 software (Olympus SIS, Münster, Germany) enabling image-capturing and image-processing.

Scanning was performed using the Scan^R acquisition module. Using a 40× objective (UPLSAPO40x2 U, Olympus, Hamburg, Germany), 241 non-overlapping fields of view were processed for each well. Prior to image acquisition, samples were autofocused using the DAPI channel (Suppl. Table S2). Subsequently, for each field of view and color channel, z-stacks were acquired (10 sections, each 0.5 μm thick) with exposure times of 1 s for ZyGold and 300 ms for the other fluorophores. A maximum intensity projection of the z-stack and a background-reduced maximum intensity projection were created virtually and saved.

2.6. Image analysis

Image Analysis was performed using Scan^R 1.3.0.3 analysis. To enable precise counting of nuclei and FISH signals, the maximum intensity projections (nuclei) and background-reduced maximum intensity projections (FISH spots) archived during the scanning process were digitally processed, including background correction and smoothing (Suppl. Table S3). For quantitative image analysis, nuclei were defined as 'main objects' and FISH signals in the nucleus area were defined as four individual types of 'sub-objects'.

Detection of nuclei was performed by Scan^R's 'edge detection' operation, i.e., signal borders of the 'main objects' (nuclei) were recognized due to their contrasts (Suppl. Table S4). For each nucleus, the xy-coordinates, area and circularity were assessed. Detection of 'sub-objects' (FISH spots) was performed using intensity-thresholds and size-limits (Suppl. Table S5). Furthermore, the acceptable distance of 'sub-objects' (FISH spots) from the main object (nucleus) border was defined (Suppl. Table S6).

Quantitative assessment of nuclei with specific FISH patterns was performed by specifically counting individual gains of CEP3 (red), CEP7 (green) and CEP17 (aqua) signals (⩽2, >2, =3, =4) and their combinations in all cells of the urinary sediment. In addition, homozygous and heterozygous losses of 9p21 signals (gold) were included into the analysis. Overall, we simultaneously gated 51 different FISH patterns. Gates were defined by 'regions', i.e., intervals of variables whose numeric values were automatically assessed during analysis, e.g., the area and the circularity of the 'main object' (nuclei) and the count of 'sub-objects' (FISH spots) (Suppl. Tables S7 and S8). Percentages were calculated for all populations relative to the total number of 'main objects' detected in the urinary sediment. To prevent the misinterpretation of background signals or contamination of the urinary sediment as nuclei, only 'main-objects' containing at least one signal of CEP3, 7 and 17 each were considered as 'nuclei'. Furthermore, restrictive gating was applied regarding area and circularity. We empirically determined a circularity of 0.9–1.15 and a size of 750–4100 pixels to adequately indicate nuclei in urinary cells. To account for potential cancer cells in the urine sediment, larger cells (4101–8000 pixels) were also included in the analysis. For larger cells, only round shapes with circularity factors ranging between 0.95 and 1.05 were analyzed, thus excluding data acquisition in case of cellular overlaps.

In addition to fully automatic gating, a facultative exclusion of objects from automatic analysis was carried out in order to verify our automated approach. For this purpose, we performed an 'expert-supervised' analysis approach to exclude objects that would create suspicious FISH patterns, e.g., due to obvious staining artifacts causing erroneous counting of nuclei or FISH spots and inaccurate detection of the main object's borders. Therefore, we viewed all galleries of the 'nuclei' and manually assigned artifacts to the region 'Excluded from analysis' (Suppl. Table S7).

2.7. Statistics

For each sample, cells harboring a specific FISH pattern were counted and expressed as a percentage of all 'nuclei'. To compare the pattern frequencies between BC patients and PCs, pairwise Wilcoxon rank sum tests were calculated for the automated and the supervised approach at a 2-tailed $\alpha = 0.05$ level of statistical significance. In addition, ROC curves were constructed and the areas under the curves (AUCs) were determined. Only FISH patterns that had AUCs which did not include 0.5 in their 95% confidence intervals in the automated analysis were considered significant and further evaluated. Exemplary cutoff values were calculated for selected FISH patterns to distinguish cancer patients from healthy individuals. Calculations were performed with SAS/STAT and SAS/IML version 9.3 (SAS Institute Inc., Cary, NC, USA). Data were plotted using GraphPadPrism software, version 5 (Graph Pad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Batch scanning and quantification of FISH signals in urinary cells

Our programmed scanning of FISH specimens and the automatic acquisition and archiving of photographs overcomes fluorescence bleaching due to redundant light exposure as a potential source of error in FISH diagnostics. In addition, a more flexible laboratory time-management for both technician and observer is achieved by uncoupling the processes of fluorescence microscopy and the subsequent evaluation using image analysis. Compared to the conventional set-up, our newly developed slide-loading device with multiple wells on a single slide reduces operating time by enabling batch-processing of 32 wells in a single step.

Automated analysis was successfully programmed to detect FISH signals. For CEP3, 7 and 17, two reasonably sized bright signals were obtained that could be easily distinguished from the background (Fig. 2). In contrast, the signals generated by LSI9p21 were small and could hardly be discriminated from the background by signal intensity or size. Weak LSI9p21 signals were also reported for the Abbott probes in a previous publication [19] and may be attributed to the smaller size of locus-specific compared to centromere-specific probes.

The conventional UroVysion™ begins the evaluation with nuclei displaying abnormal morphologies suggestive of cancer (e.g., manifested in nucleus augmentation or inhomogeneous DAPI staining) [7,14]. Nevertheless, as chromosomal aberrations are early events in carcinogenesis and precede morphological alterations [5,20], it is necessary to analyze the whole slide (regular appearing and morphologically altered cells) before reporting a negative diagnostic finding. Specimen-wide evaluation in manual UroVysion™, however, demands an average evaluation time of 20 min per specimen with a well-trained investigator [21] and is dependent on the number of cells on the slide. Therefore, several researchers restricted their investigation to a maximum number of 100 nuclei [17] or suspicious nuclei only [16]. Also initial automation approaches relied on the pre-selection of morphologically anomalous cells [9,13,22] or confined the number of analyzed cells to a maximum of 2000 [19]. To our knowledge, the system described here is unique, in that it facilitates the quantitative assessment of FISH patterns in all the nuclei present in a target

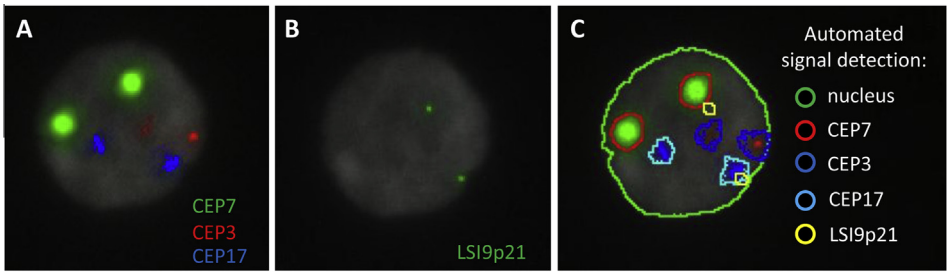

Fig. 2. Automated signal detection in a urinary specimen obtained from a control individual. Images were composed from the virtual channels. As the software only allows the simultaneous display of four color channels (including DAPI), two screenshots were necessary to display all signals. (A) CEP3, 7 and 17 signals, (B) LSI9p21 signal. The software also did not allow the free choice of colors thus the LSI9p21 signal is displayed in green rather than gold. (C) Automated detection of the nucleus and all FISH spots.

well. As this approach increases the chance of detecting hidden BC lesions, our assay might have the potential to improve diagnosis of BC by automated FISH analysis. Furthermore, quantitative analysis of all nuclei on a slide, in combination with automatic signal counting, provides the opportunity to reassess established evaluation criteria and expand the catalog of FISH patterns indicative of BC.

3.2. Assessment of probe signal-counts

To evaluate the reliability of our newly developed automated FISH spot detection assay, we investigated the distribution of CEP-gains and LSI9p21-losses as prominent features of BC in our pilot collective using both, the fully automated assay and the expert-supervised approach excluding doubtful objects from the analysis. Both approaches were applied to the complete sample set obtained from all individuals (BC and PC individuals). Therefore, the potential impact of artifacts in the fully automated assay (e.g., staining artifacts) on the discriminative power of every FISH pattern could be studied.

The expert-supervised approach revealed CEP signal-gains (CEP3 or 7 or 17 > 2) to be more frequent in urinary cells from BC patients than PCs (Fig. 3A). The significance of these differences was retained in the fully-automated analysis, thus indicating only minor influences of artifacts on the resulting percentage of aneuploid cells (Fig. 3C). These minor influences are especially true for the frequencies of gains in CEP 3 and 7 that scattered similarly in both approaches and only had slightly higher *p*-values for the BC/PC-comparison in the fully-automated evaluation.

In contrast to gains in CEP signals, both homo- and heterozygous losses of LSI9p21 displayed no differences between BC patients and PCs in both approaches (Fig. 3B and D). As deletions of LSI9p21 were described to be more frequent in low-stage and non-invasive tumors [5], our results might be due to the predominance of invasive neoplasms in our BC collective. However, no differences in LSI9p21-losses between the two groups may also be attributed to the small and weak LSI9p21 signals themselves thus making it generally difficult to distinguish LSI9p21 signals and background. In summary, the fully-automated assay showed a good coherence with the expert-guided analysis.

3.3. Automatically assessed FISH patterns of potential diagnostic value for bladder cancer

To verify whether automatically quantified FISH patterns would distinguish BC patients from controls and which pattern would discriminate best, we performed ROC-analysis and ranked all

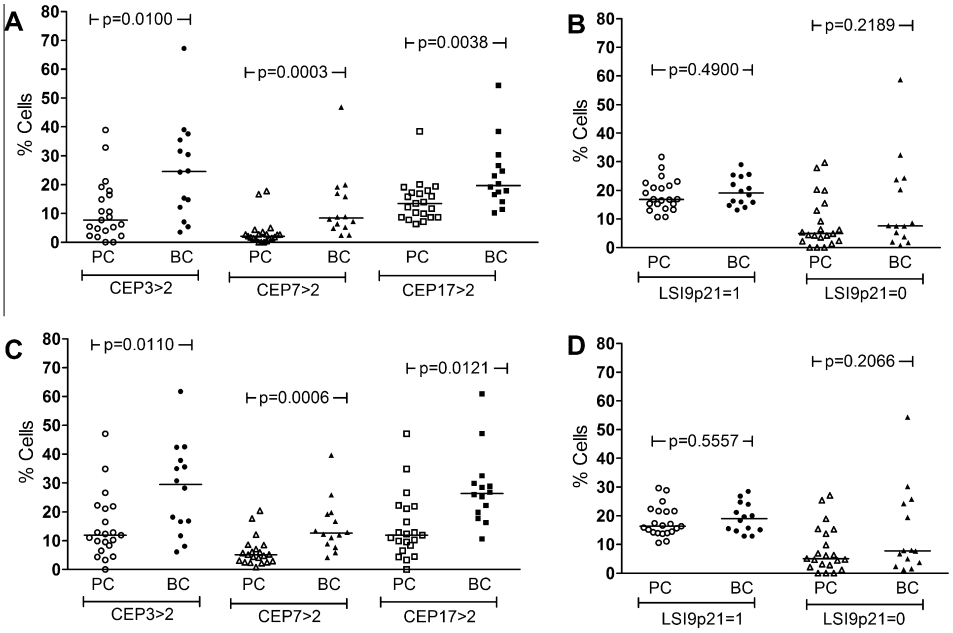

Fig. 3. Fraction of cells with gains in CEP3, 7 and 17 and hetero- or homozygous losses of LSI9p21 for the expert-guided assay (A and B) and the fully automated assay (C and D). Scatter-plots display individual data points and median values. The *p*-values are given for bladder cancer patients (BC) compared to population controls (PC).

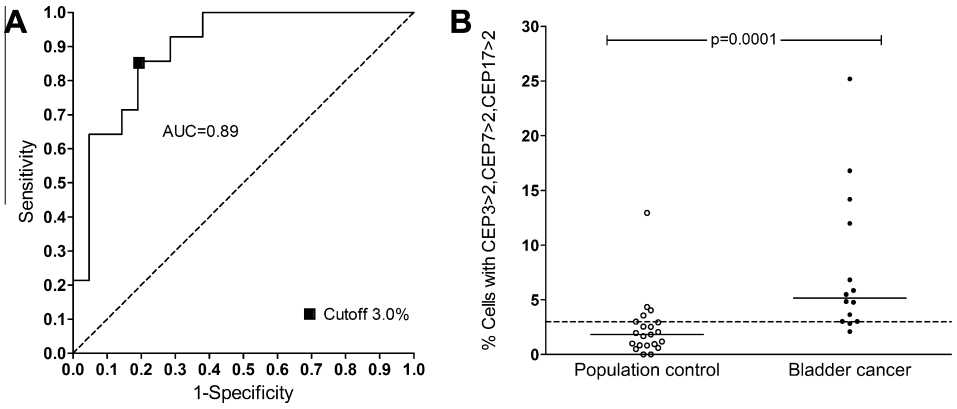

Fig. 4. ROC-curve (A) and scatter-plots (B) of the fully automated approach for CEP3 > 2 and CEP7 > 2 and CEP17 > 2. Data are shown for bladder cancer patients compared to population controls. A preliminary cutoff of 3% is depicted in the ROC-curve (square) and the scatter-plot (dotted line). The scatter-plots display individual data points and median values.

analyzed FISH patterns according to their AUC in the fully automated approach. Since AUCs between 0.75 and 0.8 were previously reported for FDA-approved urine-based BC biomarkers like bladder tumor antigen (BTA) and nuclear matrix protein (NMP22) [23], we selected for FISH patterns whose frequencies were significantly different between BC patients and PCs and which yielded AUCs > 0.8 in the ROC analysis.

Full automation revealed 15 populations that were significantly different between BC patients and PCs and which yielded an AUC > 0.8 in the ROC analysis. A comparison with the expert-supervised approach revealed that twelve out of these 15 FISH patterns retained their considerable and significant cancer identifying power. Similar AUC values in both approaches further supported the power of the fully automated approach (Suppl. Table S9). In line with the highest significance of CEP-gains in BC (Fig. 3A and C), CEP7-signal counts co-define the FISH patterns best-discriminating for BC in our pilot collective (Patterns 1–4 in Suppl. Table S9). As previously reported for UroVysion™ [7], our data also suggest that additional gains in CEP17 and 3 further increase the AUC for BC-detection. Consequently, the highest AUC value obtained by full automation was 0.89 for CEP3 > 2, CEP7 > 2, and CEP17 > 2. When using a 3% cutoff for this triple polysomy, BC could be detected by fully automated FISH spot quantification with a sensitivity of 86% and a specificity of 81% (Fig. 4).

In summary, we established a unique method for a fully automated and objective assessment of FISH signals that enables the quantification of complex FISH patterns in all nuclei of the urinary sediment. Furthermore, we designed a new technique for sample application that considerably reduces scanning and operating time by allowing batch sampling. Comparison of the fully automated assay with an expert-guided evaluation of the FISH patterns suggests a limited impact of artifacts on the resulting quantities of assessed nuclei-populations thus supporting the objectivity and reliability of the fully automated assay. Our pilot study revealed that automatic recognition of CEP3, 7, 17 and LSI9p21 FISH signals could identify BC by several FISH patterns with the best discriminative patterns including gains in CEP7. Our automated analysis encourages its use to identify the most reliable diagnostic FISH pattern and to establish reliable diagnostic cutoffs in future large-scale studies with well characterized specimens including a broad variety of different types of BC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.137.

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