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PHOTOPROBE® biotin: An alternative method for labeling archival DNA for comparative genomic hybridization

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Abstract. Comparative genomic hybridization (CGH) represents a powerful method for screening the entire genome of solid tumors for chromosomal imbalances. Particularly it enabled the molecular cytogenetic analysis of archival, formalin-fixed, paraffin-embedded (FFPE) tissue. A well-known dilemma, however, is the poor DNA quality of this material with fragment sizes below 1000 bp. Nick translation, the conventionally used enzymatic DNA labeling method in CGH, leads to even shorter fragments often below a critical limit for successful analysis. In this study we report the alternative application of non-enzymatic, PHOTOPROBE® biotin labeling for conjugation of the hapten to the DNA prior to *in situ* hybridization and fluorescence detection. We analyzed 51 FFPE tumor samples mainly from the upper respiratory tract by both labeling methods. In 19 cases, both approaches were successful. The comparison of hybridized metaphases showed a distinct higher fluorescence signal of the PHOTOPROBE® samples sometimes with a discrete cytoplasm background which however did not interfere with specificity and sensitivity of the detected chromosomal imbalances. For further 32 cases characterized by an average DNA fragment size below 1000 bp, PHOTOPROBE® biotin was the only successful labeling technique thus offering a new option for CGH analysis of highly degraded DNA from archival material.

Keywords: CGH, non-enzymatic labeling, FFPE tissue

1. Introduction

CGH is based on the principle of fluorescence *in situ* hybridisation, and has been first described by Kallioniemi et al. and du Manoir et al. [1–3]. It was the first genome-wide molecular screening technique that allowed the detection of chromosomal alterations by mapping DNA imbalances on normal chromosome metaphase spreads. Hapten or fluorescence conjugated normal DNA is hybridized competitively with differentially labeled tumor DNA onto the metaphases. Overrepresentated chromosomal regions putatively harbour tumor promoting genes, e.g. proto-oncogenes, whereas deletions are suspicious for the inactivation of tumor growth inhibiting genes, e.g. suppressor genes.

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In the past, countless CGH-studies were published and enlarged our understanding about the genetics and biology of various tumors [4–7]. An essential advantage of CGH was the ability of tumor genome analysis without the requirement of vital tissue, cell cultivation and subsequent chromosome preparation. Initially DNA from frozen tissue was investigated, but soon the analysis was extended to formalin-fixed, paraffin-embedded tissues offering the possibility to analyze rare tumor entities from archival tissue banks [8]. The reduced quality of DNA, acquired from paraffin-embedded tissue is well-known and regularly limited the molecular genetic analysis by CGH or PCR [9,10]. Often the DNA appears degraded, showing DNA fragments smaller than 1000 bp in the gel electrophoresis (Fig. 1).

For CGH the conjugation of DNA fragments is required, the standard procedure is enzymatic labeling by nick translation which is based on the incorporation of modified nucleotides by the DNA-polymerase

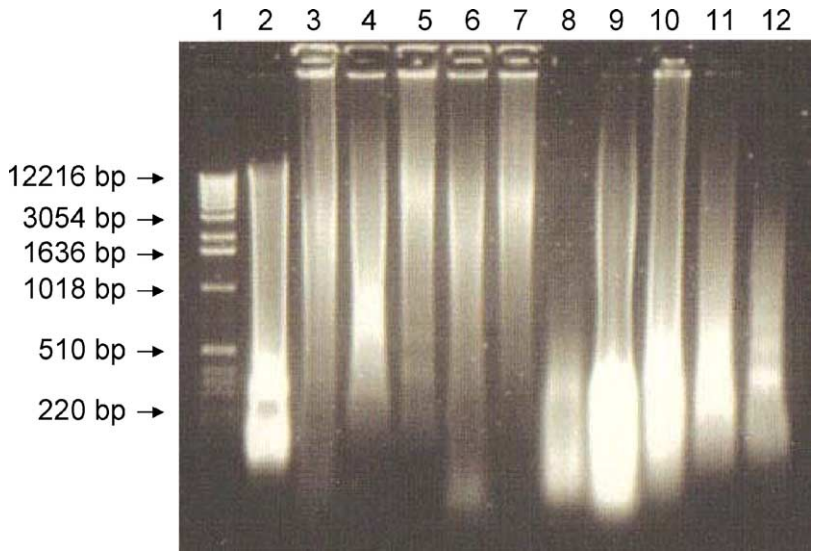

Fig. 1. Representative agarose gel electrophoresis of extracted DNA before (lanes 2–10) and after labeling and DNase treatment (lanes 11 and 12). All cases show a smear indicative for DNA degradation. The samples in lanes 2 and 3 could be successfully analyzed using nick translation and PHOTOPROBE® biotin. Since there was a high proportion of DNA larger than 1000 bp, these samples were submitted to DNase treatment after PHOTOPROBE® biotin labeling (lanes 11 and 12). They were then successfully hybridized and correspond to the results in Figs 2B and 2D. Sample in lanes 4 and 5 could only be successfully hybridized after PHOTOPROBE® biotin treatment (see Figs 2E and 2F), similar to those in lanes 6, 7 and 10. Cases in lanes 8 and 9 are representative for those that could not be analyzed by either of both labeling methods.

after the introduction of nicks into the double strand by the DNase. The DNase decreases the size of the DNA which is a desirable effect for high molecular fragments from fresh frozen tissue because it leads to the optimal strand size for hybridization between 300 and 2000 bp. For low molecular DNA from FFPE tissue, however, the fragment length will decrease below the critical limit of 300 bp causing a weak or undetectable fluorescence signal.

As solution for this dilemma we used a non-enzymatic labeling system which is based on the activation of a biotin analogue by heat or irradiation [11]. The employed, commercially available system is based on an aryl azide derivative of biotin with a positively charged spacer arm between the biotin and azide group (PHOTOPROBE® biotin, *Vector Laboratories*). Only a short step of exposition towards heat or UV-light is required for labeling the DNA which occurs not base-specific. To our knowledge this is the first study about the use of PHOTOPROBE® biotin for labeling DNA in CGH analysis.

2. Material and methods

2.1. Tissue samples

The DNA originated from a collection of FFPE tissue blocks of 51 malignant tumors of the respiratory tract (42 sinunasal, intestinal type adenocarcinomas, 3 squamous cell carcinomas, 1 esthesioneuroblastoma, 1 small cell neuroendocrine carcinoma, 1 undifferentiated carcinoma, 1 polymorphous low-grade adenocarcinoma, 1 synovialoma and 1 small cell lung carcinoma) from patients being operated throughout Germany between the years 1998 and 2003. No information about the tissue processing, in particular the time and specific procedures of formalin fixation were available. Since the majority of samples originated from the paranasal sinuses often including bone particles, additional decalcification by hydrochloric acid or other potentially DNA damaging agents might have been applied.

2.2. DNA extraction

The tumor DNA was isolated from several 20 μm-thick tissue sections after microscopic evaluation of the H&E-stained paraffin-embedded specimen. Only samples with at least 70% tumor content were accepted for DNA preparation. Samples with less tumor proportion were micro-dissected to ensure optimal detection of chromosomal gains and losses. The paraffin sections were first de-waxed in xylene and washed in ethanol twice for 10 min each at room temperature. Dried samples were digested in 800 μl DNA isolation buffer

(50 mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween-20) containing proteinase K to a final concentration of 1 mg/ml and incubated overnight at 55°C with shaking. After extractionwith phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and re-suspension in TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) the quality of DNA was controlled by measuring optical density using a photometer (Genequant II, *Pharmacia Biotech*) and by gel electrophoresis with a DNA size marker for fragment length estimation (Fig. 1).

2.3. DNA labeling

For labeling with PHOTOPROBE® biotin we used thermal coupling, complied with the instructions recommended by the protocol of *Vector laboratories*. Using a 0.5 ml Eppendorf tube each specimen consisted of 10 μl DNA solution in a concentration of $\leqslant 0.5$ μg/μl diluted in TE-buffer, mixed with an equal volume of PHOTOPROBE® biotin and overlaid with 10 μl mineral oil. We performed this step under light protected conditions to avoid loss of biotin photoreactivity. Immediately afterwards the probes were transferred into a heating block preheated at 95°C and incubated for 30 min. Subsequent to thermal coupling the probe was added to a volume of 80 μl with bi-distilled water. Then, 80 μl 0.1 M Tris, pH 9.5 and 160 μl of 2-butanol were added, followed by vigorous vortexing. For separating the phases we centrifuged by 13000g for 10 min, afterwards the upper 2-butanol phase was removed as completely as possible and discarded for maximal removal of residual biotin. This washing step was repeated once. The 2-butanol washed out the PHOTOPROBE® biotin and reduced the aqueous phase to approximately 40 μl. A careful washing influences directly a successful hybridization with minimal background and may compensate for the application of DNA purification columns which is recommended by *Vector laboratories* for *in situ* hybridization.

For precipitating the biotinylated DNA, 10 μl of 10 M ammonium acetate, 2 μl of 1 M $MgCl_2$ and 125 μl of -20°C ethanol (100%) were added, mixed and incubated at -20°C for 30 min followed by centrifugation at 13000g and 4°C for at least 15 min. A red-brown pellet confirmed the successful biotin-conjugation which was washed with 70% ethanol followed again by centrifugation for 3 min. The pellet was air-dried and re-suspended overnight at 4°C in 40 μl bi-distilled water.

After labeling, the DNA was re-evaluated by agarose gel electrophoresis. In case of DNA samples with a proportion of high molecular DNA (bulk of DNA with fragment size above 1000 bp), the PHOTOPROBE® labeled specimens were treated with DNase (stock solution 3 mg/ml, final dilution of 1 : 100000) for 20 min at 15°C to achieve an optimal fragment length for hybridization.

2.4. Comparative genomic hybridization

CGH was performed as previously described [12, 13]. Detailed protocols are also available at the website http://amba.charite.de/cgh/. In brief, 1 μg of labeled tumor and 1 μg of labeled normal DNA were ethanol-precipitated in the presence of 30 μg Cot-1 human DNA and 10 μg herring sperm DNA, then dried and re-suspended in 5 μl formamide for 20 min at 37°C following the addition of 10 μl master mix (4x SSC/20% dextransulfate). The DNA was then denatured at 77°C for 5 min and allowed to re-anneal at 37°C for 90 min. Slides containing target metaphase chromosomes from healthy donor prepared from peripheral, PHA-stimulated T-lymphocytes (*Vysis*) were denatured for 5 min at 73°C in 70% deionised formamide in 2x SSC (pH 7.0–7.5), subsequently dehydrated in 70%, 90% and 100% ice-cold ethanol and air-dried. The DNA probe mixture was applied to the denatured metaphase chromosomes under an 18 × 18 mm cover slip, sealed with rubber cement and hybridized at 37°C in a water bath. After 3 days, the slides were washed for 3 × 3 min in formamide/2xSSC (1 : 1) at 37°C and 3 × 2 min in 0.1x SSC at 60°C, shortly stored in 4x SSC/0.1% Tween-20 until blocking with BSA for 20 min in a humid chamber at 37°C. The detection mixture was prepared by mixing 10 μl FITC (fluorescein–avidin, Vector Laboratories) and 9 μl TRITC (anti-dig-rhodamin, Roche Diagnostics) in 1 ml 3% BSA, 125 ml were used for each slide, covered with a 24 × 60 mm cover slip and incubated for 30 min in a humid chamber at 37°C. After washing for 3 × 3 min in 4x SSC/0.1% Tween-20 the chromosomes were counterstained with DAPI, covered in 35 μl DABCO solution using a 24 × 60 mm cover slip and stored at 4°C under light protection.

For image acquisition we used a cooled charge-coupled device camera (Photometrics) mounted on an epifluorescence microscope (Zeiss Axiophot) with filter equipment detecting DAPI, FITC and TRITC. Preferably we have chosen complete, well spread metaphases with less overlapping chromosomes and without neighbouring, intensively shining objects like nuclei or fluorochrome particles.

For digital image analysis we used custom-made CGH software in all cases [14]. At least 15 metaphases of each case were analysed and average ratio profiles were calculated to suppress random changes. Details regarding the digital image analysis we have been published before and are available at the website http://amba.charite.de/cgh/. Chromosomal imbalances were scored by fluorescence ratio profiles with 99% confidence intervals deviating significantly from the normal ratio value 1.0 [12,13].

3. Results and discussion

In total, 51 tumor samples were labeled with biotin using nick translation as well as PHOTOPROBE® biotin and successfully hybridized. Nineteen of the nick translated samples could be analyzed by CGH. The remaining 32 cases could only be successfully hybridized after non-enzymatic biotin labeling. Additional 18 samples did not yield sufficient fluorescence signals after hybridization.

Representative samples of all three subgroups are shown in Fig. 1. Agarose gel electrophoresis was an appropriate method to test the fragment size and thus the quality of DNA. In general, samples with highly degraded DNA with a bulk of fragments smaller than 500 bp length were difficult to hybridize successfully. However, fragment size was not the only criterion that could predict the hybridization result. The sample in lane 2 could be analyzed despite the fact that it harboured small DNA fragments. It originated from an autopsy case of a small cell carcinomas which was kept as frozen tissue until DNA extraction. In contrast, all other DNA samples were extracted from FFPE tissue. Similarly, samples in lanes 4, 5, 6, 7 and 10 could only be analyzed by using PHOTOPROBE® biotin although the DNA appeared similar in the agarose gel to the sample in lane 3 which was also successful after the classical nick translation.

Comparison of both methods showed almost background-free results for the enzymatic-labeled probes. In PHOTOPROBE® hybridizations a higher frequency of cytoplasm background signals was observed which was presumably due to the presence of unbound PHOTOPROBE® biotin traces within the hybridization solutions. High quality, cytoplasm-free metaphases are therefore recommended for this non-enzymatic labeling procedure. Additional extraction steps with 2-butanol to reduce the amount of residual biotin in the aqueous phase should be considered and may avoid the use of DNA purification columns as recommended by the manufacturer.

Even without column purification we could achieve high quality CGH results as exemplified in Fig. 2. It represents hybridizations with both labeling methods (Fig. 2A,C, PHOTOPROBE® biotin, Fig. 2B,D, nick translation) and indicates a high concordance, thus suggesting no significant disturbance of specificity and sensitivity of Photoprobe labeling in the detection of DNA gains and losses.

The CGH analysis of a Photoprobe labeled sinonasal adenocarcinoma that could not be analyzed after nick translation is shown in Fig. 2E. For control-versus-control experiments we used PHOTOPROBE® biotin-conjugated DNA of paraffin-embedded, non-cancerous polyposis nasi tissue which was co-hybridized with normal DNA labeled with digoxigenin by nick translation (Fig. 2F). No chromosomal imbalances were detected.

The influence of the quality of DNA from FFPE tissue on molecular genetic analysis by PCR or CGH is well documented [9,10]. Shibata et al. investigated fixatives other than formalin like Zenker's, Bouin's and B-5 as well as the fixation times thereby establishing parameters that influence the evaluation of paraffin-embedded tissue [9]. The proportion of highly degraded tumor samples of our collective is related to the fact that the specimens originated from diverse pathology institutes nationwide and not primarily university institutes, the samples were processed routinely without the initial assumption of later genetic analysis. No information about the tissue processing, in particular the time and specific procedures of formalin fixation like the use of buffered formalin were documented. Since the majority of samples originated from the paranasal sinuses often including bone particles, additional decalcification by hydrochloric acid or other potentially DNA damaging agents might have been applied.

Alers et al. described an alternative method using a bi-valent platinum compound called universal linkage system (ULS) for labeling degraded DNA from paraffin-embedded tumor tissues. The substance preferably binds to the guanine groups, in contrast to PHOTOPROBE® biotin which has no base-specific affinity. The unequal distribution of guanine within the genome is well known; the bright chromosomal bands in G-banding are enriched in GC nucleotides and also genes. This pattern could induce CGH artefacts, thus we have chosen PHOTOPROBE® biotin on the assumption that binding to the DNA occurs more regu-

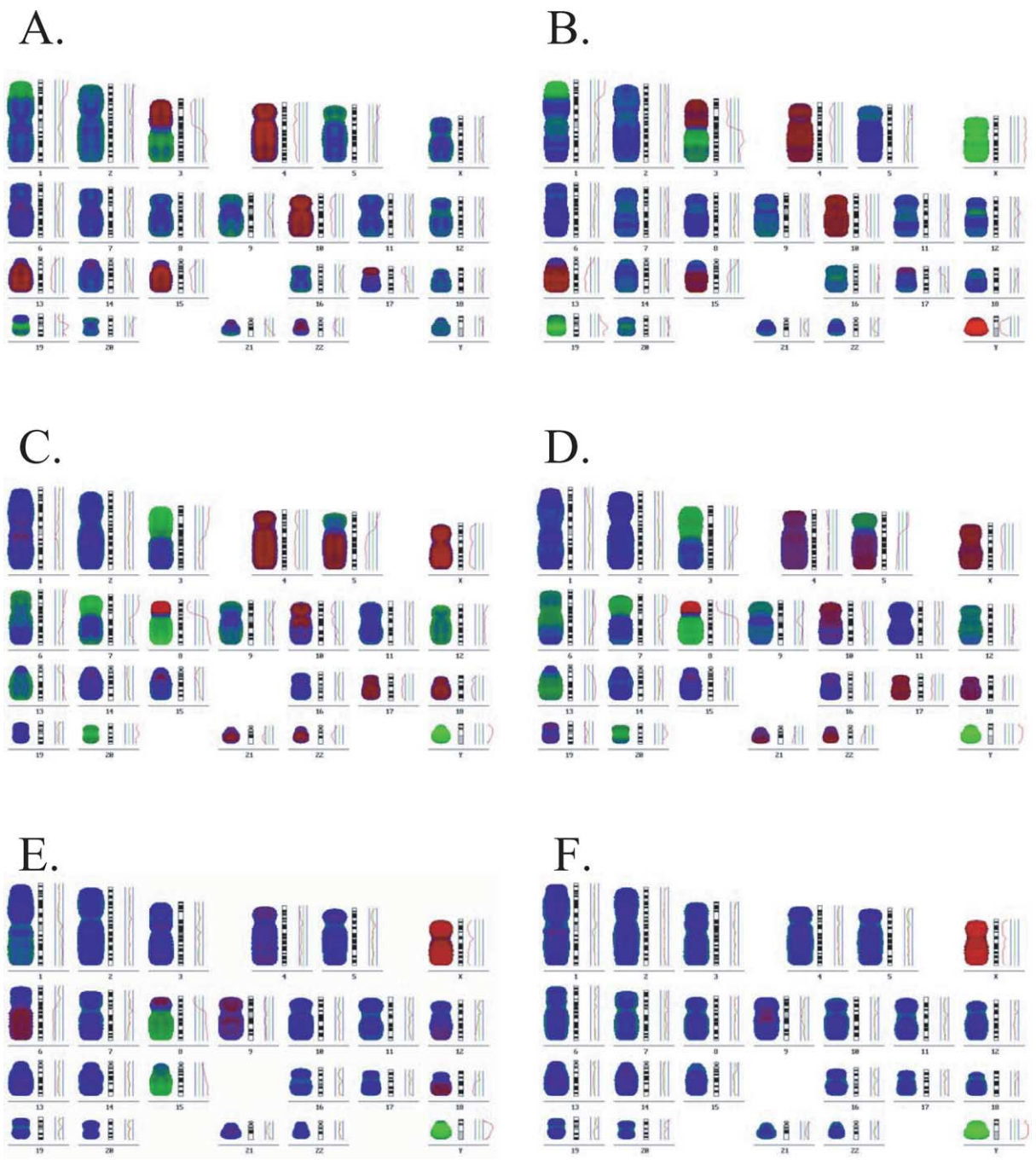

Fig. 2. CGH sum karyograms were created after analysing PHOTOPROBE® and nick translation labeled tumor DNA from a small cell lung cancer (A,B) and a sinonasal, intestinal type adenocarcinoma (C,D). Additionally, a sinonasal, polymorphous low-grade adenocarcinoma that could not be analyzed after nick translation (E) and non-cancerous polyposis nasi tissue (F) as control hybridization after PHOTOPROBE® labeling are shown. Chromosomes from 15 metaphases were analyzed for each hybridization. The PHOTOPROBE® biotin (A,C) and nick labeled samples (B,D) showed the same aberrations. Typical imbalances of sinonasal adenoacarcinoma were gains of 1q, 8q, 15 and losses of chromosome 4, 6q, 8p, 9, 12q21-qter, 18q and 21q21 (E, PHOTOPROBE®). The control hybridization showed no imbalances and was performed with PHOTOPROBE® labeled DNA prepared from polyposis nasi tissue and normal, nick translation digoxigenin-labeled DNA from peripheral blood cells from a healthy donor (F).

larly. ULS-labeled DNA can be used immediately for hybridization, but it also carries the potential to induce an increased background signal. They suggested the application of Qiagen columns for DNA purification to ensure better hybridization results with decreasing unspecific background staining [10].

Conventional CGH is still a powerful tool for the detection of aneuploidy and specific chromosomal imbalances with a sensitivity of about 10 Mb for deletions and few kb for amplifications [15]. Array based techniques have meanwhile been developed [16,17] and recently the non-enzymatic ULS technique has been described for array CGH [18].

We conclude that degraded DNA being unsuitable for nick translation can be successfully labeled and hybridized using PHOTOPROBE® biotin thus increasing the potential to analyse archival tissue samples by CGH.

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References

[1] A. Kallionemi, O.P. Kallionemi, D. Sugar, D. Rutovitz, J.W. Gray, F. Waldman and D. Pinkel, Comparative genomic hybridisation for molecular cytogenetic analysis of solid tumors, *Science* **258** (1992), 818–821.

[2] O.P. Kallioniemi, A. Kallioniemi, J. Piper, J. Isola, F.M. Waldman, J.W. Gray and D. Pinkel, Optimizing comparative genomic hybridisation for analysis of DNA sequence copy number changes in solid tumors, *Genes Chromosomes Cancer* **10** (1994), 231–243.

[3] S. du Manoir, M.R. Speicher, S. Joos, E. Schrock, S. Popp, H. Dohner, G. Kovacs, M. Robert-Nicoud, P. Lichter and T. Cremer, Detection of complete and partial chromosome gains and losses by comparative genomic in situ hybridization, *Human Genetics* **90**(6) (1993), 590–610.

[4] C. Schleger, N. Arens, H.U. Zentgraf, U. Bleyl and C. Verbeke, Identification of frequent chromosomal aberrations in ductal adenocarcinoma of the pancreas by comparative genomic hybridization (CGH), *Journal of Pathology* **191** (2000), 27–32.

[5] S.C. Stocks, N. Pratt, M. Sales, D.A. Johnston, A.M. Thompson, F.A. Carey and N.M. Kernohan, Chromosomal imbalances in gastric and esophageal adenocarcinoma: Specific comparative genomic hybridization-detected abnormalities segregate with junctional adenocarcinomas, *Genes Chromosomes Cancer* **32** (2001), 50–58.

[6] I. Petersen, S. Petersen, U. Bockmuhl, A. Schwendel, G. Wolf and M. Dietel, Comparative Genomische Hybridisierung an Bronchialkarzinomen und ihren Metastasen, *Verh. Dtsch. Ges. Path.* **81** (1997), 297–305.

[7] A. Goeze, K. Schluns, G. Wolf, Z. Thasler, S. Petersen and I. Petersen, Chromosomal imbalances of primary and metastatic lung adenocarcinomas, *Journal of Pathology* **196** (2002), 8–16.

[8] M.R. Speicher, S. du Manoir, E. Schrock, H. Holtgreve-Grez, B. Schoell, C. Lengauer, T. Cremer and T. Ried, Molecular cytogenetic analysis of formalin-fixed, paraffin-embedded solid tumors by comparative genomic hybridization after universal DNA-amplification, *Human Molecular Genetics* **2**(11) (1993), 1907–1914.

[9] D. Shibata, Extraction of DNA from paraffin-embedded tissue for analysis by polymerase chain reaction: New tricks from an old friend, *Human Pathololgy* **25** (1994), 561–563.

[10] J.C. Alers, J. Rochat, P.J. Krijtenburg, H. van Dekken, A.K. Raap and C. Rosenberg, Universal linkage system: An improved method for labeling archival DNA for comparative genomic hybridisation, *Genes Chromosomes Cancer* **25** (1999), 301–305.

[11] A.C. Forster, J.L. McInnes, D.C. Skingle and R.H. Symons, Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin, *Nucleic Acids Research* **13** (1985), 745–761.

[12] I. Petersen, M. Bujard, S. Petersen, G. Wolf, A. Goeze, A. Schwendel, H. Langreck, K. Gellert, M. Reichel, K. Just, S. du Manoir, T. Cremer, M. Dietel and T. Ried, Patterns of chromosomal imbalances in adenocarcinoma and squamous cell carcinoma of the lung, *Cancer Research* **15;57**(12) (1997), 2331–2345.

[13] I. Petersen, Comparative genomic hybridization of human lung cancer, *Methods in Molecular Medicine* **75** (2003), 209–237.

[14] K. Roth, G. Wolf, M. Dietel and I. Petersen, Image analysis for comparative genomic hybridization based on a karyotyping program for Windows, *Analytical Quantitative Cytology and Histology* **19**(6) (1997), 461–474.

[15] M. Bentz, A. Plesch, S. Stilgenbauer, H. Dohner and P. Lichter, Minimal sizes of deletions detected by comparative genomic hybridization, *Genes Chromosomes Cancer* **21**(2) (1998), 172–175.

[16] S. Solinas-Toldo, S. Lampel, S. Stilgenbauer, J. Nickolenko, A. Benner, H. Dohner, T. Cremer and P. Lichter, Matrix-based comparative genomic hybridization: Biochips to screen for genomic imbalances, *Genes Chromosomes Cancer* **20**(4) (1997), 399–407.

[17] D. Pinkel, R. Segraves, D. Sudar, S. Clark, I. Poole, D. Kowbel, C. Collins, W.L. Kuo, C. Chen, Y. Zhai, S.H. Dairkee, B.M. Ljung, J.W. Gray and D.G. Albertson, High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays, *Nature Genetics* **20**(2) (1998), 207–211.

[18] A.K. Raap, M.J. van der Burg, J. Knijnenburg, E. Meershoek, C. Rosenberg, J.W. Gray, J. Wiegant, J.G. Hodgson and H.J. Tanke, Array comparative genomic hybridization with cyanin cis-platinum-labeled DNAs, *Biotechniques* **37**(1) (2004), 130–134.