The tissue is the issue: improved methylome analysis from paraffin-embedded tissues by application of the HOPE technique

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Alterations in the DNA methylome are characteristic for numerous diseases and a typical hallmark of cancer. Therefore, DNA methylation is currently under investigation in research labs and has also entered diagnostics. Recently, protocols like the BeadChip technology have become commercially available to study DNA methylation in an array format and semiquantitative fashion. However, it is known that fixation of the sample material with formalin prior to BeadChip analysis can affect the results. In this study we compared the influence of fixation on the outcome of BeadChip analysis. From six patients each a lung cancer tissue sample and a corresponding tumor-free lung tissue sample were collected. The samples were separated into three pieces. One piece of each sample was fixed with formalin, another one by the non-cross-linking HOPE technique (Hepes-glutamic acid buffer mediated Organic solvent Protection Effect). Subsequently, both became paraffin embedded. As a reference, the remaining third piece was cryopreserved. In addition we used three adenocarcinoma cell lines (H838, A549, and H1650) to validate the results from patient tissues. We show that using the HOPE technique instead of formalin largely prevents the introduction of formalin-fixation related artifacts. An ANOVA analysis significantly separated HOPE- and cryopreserved from formalin-fixed samples (FDR < 0.05), while differences in the methylation data obtained from HOPE-fixed and cryopreserved material were minor. Consequently, HOPE fixation is superior to formalin fixation if a subsequent BeadChip analysis of paraffin-embedded sample material is intended.

INTRODUCTION

Current research approaches with human tissues have to deal with limitations of the material. The use of archived patient material allows screening for valuable biomarkers or validation of those in complex cellular systems. The vast majority of archived patient material still is formalin-fixed and paraffin-embedded (FFPE), which delivers an excellent histological morphology and allows large-scale screening projects. As formalin is a cross-linking agent, antigenic structures are usually masked and therefore epitope-retrieval is necessary for antibody-based applications.[1,2] Additionally, a major drawback of FFPE specimens is the degradation of nucleic acids,[3] which influences gene expression analysis or other DNA/RNA-based techniques.[4] Hence, much efforts have been undertaken to overcome the obstacles of FFPE tissues and allow modern genetic[5] and proteomic[6] assays. Therefore, investigators have to face a trade-off between the applicability of different techniques with respect to both, the available amount of material and the way of tissue preservation. Recent developments have successfully demonstrated the feasibility of applying diverse techniques such as 2D proteome analysis[7] or qPCR and transcriptome analysis[8,9] from the same paraffin-embedded specimen block by application of the HOPE fixation.[10] Using HOPE for biomaterial preservation

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offers a multitude of read-out techniques with little nucleic acid degradation[11,12] and the possibility of immuno-phenotyping the sample without epitope-retrieval.[13]

Here we present an application of the well-described HOPE technique[14,15] to the field of DNA methylation research. DNA methylation is a key player for the establishment of tissue-specific gene expression patterns and cell differentiation,[16] and aberrant DNA methylation has been reported in numerous diseases, in particular in cancer.[17] It has been shown before that formalin fixation of the samples or the use of DNA extracted from FFPE material can result in differences between frozen and FFPE material in subsequent array-based DNA methylation analyses.[18] This is probably caused by DNA cross-linking and fragmentation of the DNA due to formalin treatment and might interfere with whole-genome amplification steps. This holds also true for DNA methylation analyses performed by the Infinium HumanMethylation450K BeadChip.[18] This commercially available array which allows the parallel analysis of more than 450 000 CpG loci has become a valuable tool for many researchers to get an overview about the DNA methylome and is currently used in numerous research projects.[19] Here we tested the usability of HOPE-fixed, paraffin-embedded lung tissues for array-based DNA methylation analysis and compared the outcome with data obtained from cryopreserved and FFPE material.

MATERIALS AND METHODS
Cell Lines
Human adenocarcinoma cell lines A549, H838, and H1650 (a kind gift from Prof Dr Ursula Klingmüller, German Cancer Research Center, Heidelberg) were cultured in DMEM high glucose medium (PAA, Pasching, Austria) supplemented with 10% FCS, 1% Penicillin/Streptomycin, 1% L-glutamine, 1% sodium-pyruvate and 1% non-essential amino acids (all from PAA, Pasching, Austria) in an incubator with 37 °C and 5% CO_2. For cell-block production, the culture medium was removed and the cells washed with PBS. Cells were detached using Trypsin–EDTA (PAA), washed with PBS and equal amounts of cells separated for fixation. Paraffin-embedding of cells as well as cell-block production was conducted as described in the study by Marwitz *et al.*[8]

Tissue Processing, Fixation, and Ethics
Matched tissue samples (tumor-free lung and tumor) were obtained from six patients undergoing pneumectomy or lobectomy at the LungenClinic Grosshandorf in the course of surgical treatment of previously diagnosed lung cancer. The freshly removed tissues were kept at 4 °C until processing as following: From each tissue (tumor and corresponding tumor-free lung), three specimens of comparable size were macroscopically obtained by a trained pathologist and preserved applying HOPE fixation and paraffin-embedding as described elsewhere[10] as well as standard formalin-fixation with subsequent paraffin-embedding. In short, HOPE-fixed

samples were removed from the protective HOPE solution and dehydrated by incubation (6 × 1 h) in 100% acetone at 4 °C. After dehydration, the tissue samples were immersed in low-melting Paraffin (52–54 °C; Medite, Burgdorf, Germany) overnight and used to prepare paraffin blocks with low-melting paraffin. For formalin fixation, 4% neutral-buffered formalin was used overnight. Next day, the tissues were dehydrated by increasing series of alcohol (70% 1 h, 80% 1 h, 2 × 96% 1 h, 3 × 100% 1 h, xylene 1 h, xylene 45 min.) and immersed in paraplast (56 °C melting point; McCormick Scientific, VWR, Darmstadt, Germany) for a total of 3.5 h in a Shandon pathcentre (ThermoScientific, Karlsruhe, Germany). Tissues for cryo-fixation were snap-frozen in liquid nitrogen and kept at − 80 °C until further use. The use of patient materials was approved by local ethics committee of the University of Lübeck (AZ 12-220). Table 1 summarizes patient data.

DNA Isolation from Micro-Dissected Tissue Samples
For enrichment with tumor cells, H&E-stained slides of formalin- and HOPE-fixed samples were micro-dissected under a microscope and the tumor cells transferred to DNA isolation using Qiagen QIAmp Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. In short, 5-μm-thick sections of each paraffin-block were cut on a microtome (SM 2000 R, Leica, Wetzlar, Germany), mounted on SuperFrost slides (Menzel Gläser, Braunschweig, Germany), deparaffinized by xylene and rehydrated with ethanol as following: 1 × 10 min xylene, 2 min 100% ethanol, 2 min 96% ethanol, 2 min 80% ethanol, 2 min 70% ethanol, 2 min aq.dest. The H&E-stained sections were directly used for micro-dissection without prior dehydration and addition of mounting media. Cryopreserved samples were macroscopically dissected, homogenized with liquid nitrogen in a mortar and submitted directly to DNA extraction. Purity of nucleic acids was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Mean DNA extraction yields ± s.d. are shown in Table 2.

Table 1 Summary of patient data characteristics

Sample no.	Age	Gender	Histologic diagnosis of tumor sample	TNM of tumor sample
5285	78	Female	Adenocarcinoma	pT1B, pN0, L0, V0, R0
5770	70	Male	Adenocarcinoma	pT2a, N1, V1, L1, G2, R0
15 136	73	Male	Large cell carcinoma	pT2a, pN3, G3, R0, L1, V1
15 343	59	Male	Squamous cell carcinoma	pT3, N3, G3, L1, V0, R0
15 724	66	Male	Squamous cell carcinoma	pT3, pN0, G2, R0
15 725	79	Female	Adenosquamous carcinoma	pT2a, pN0, G2, R0, L0, V0

Table 2 Summary of nucleic acid extraction yield

Fixation applied	Mean nucleic acid yield (ng/μl) $\pm$ s.d.	Mean $\pm$ s.d. 260/280 nm ratio
HOPE	Tumor-free lung: 92.1 $\pm$ 58.2; tumor: 234.7 $\pm$ 109.5	Tumor-free lung: 1.9 $\pm$ 0.2; tumor: 1.9 $\pm$ 0.1
Formalin	Tumor-free lung: 199.7 $\pm$ 32.3; tumor: 528.3 $\pm$ 207.1	Tumor-free lung: 1.9 $\pm$ 0.1; tumor: 1.8 $\pm$ 0.3
Cryo	Tumor-free lung: 35.8 $\pm$ 22.9; tumor: 162.6 $\pm$ 90.8	Tumor-free lung: 1.8 $\pm$ 0.3; tumor: 1.9 $\pm$ 0.2

DNA Methylation Screening

Bisulfite conversion of genomic DNA was performed applying the EZ DNA Methylation kit (ZymoResearch, Irvine, CA, USA) following the protocol supplied by the manufacturer. Subsequent DNA methylation analysis using the Infinium HumanMethylation450k BeadChip (Illumina Inc., San Diego, CA, USA) was performed according to the manufacturer's instruction. The HumanMethylation450K BeadChip was developed to assay more than 480 000 CpG sites in parallel.[20] DNA methylation data were processed using GenomeStudio software (v2011.1; methylation modul 1.9.0; Illumina, Inc.) applying the default settings. The intrinsic controls present on the array were used for data normalization, no further background subtraction or normalization steps were performed. All primary tissue samples as well as cell lines were processed in parallel using the same batch of arrays and kits.

Data Analysis

Loci with detection of P-values above 0.01 in at least one of the samples were excluded from further analysis. Finally, 483 238 of the 485 577 loci present on the array entered subsequent analyses. Additionally, loci with missing avg.beta values were excluded from the comparisons of the DNA methylation values of the individual patients.

GenomeStudio software was used to perform inter-sample comparisons and to calculate coefficients of determination. T-test statistics were calculated using Prism software (ver. 4.02; GraphPad Software, San Diego, CA, USA). R (http://www.r-project.org/) was used to generate box plots and to perform cluster analyses of samples. Further principal component (PCA) and hierarchical cluster analyses were performed using the OMICS Explorer 2.1 (Version 2.1;[21] Qlucore, Lund, Sweden) using DNA methylation values (average beta values) obtained from the GenomeStudio software.

Bisulphite Pyrosequencing

Bisulfite pyrosequencing was performed as described before.[22] Genomic DNA was bisulfite converted using the EpiTect Bisulfite Conversion Kit (Qiagen) according to the manufacture's recommendations. For PCR amplification, locus-specific primers were used with one primer biotinylated at the 5′ end (Supplementary Table 1). PCR products were verified by gel electrophoresis and single strands were prepared using the Vacuum Prep Tool (Biotage, Uppsala, Sweden). Pyrosequencing was performed using the Pyrosequencer ID and the DNA methylation analysis software Pyro Q-CpG 1.0.9 (Biotage).

RESULTS

Reproducibility and Quality Assessment of The Beadarray Methylation Analysis

We quantified the methylation status of 485 577 methylation sites in 36 tissue samples using the BeadArray technology.[20] The complete data set is provided at Gene Expression Omnibus (GSE51077). The median call rate on the methylation arrays was 99.95% (range 99.85–99.99%) indicating robust technical performance of the hybridization (Supplementary Figure 1). The vast majority of loci present on the array (99.53%) passed a detection with a P-value threshold of 0.01 applied to all 36 samples analyzed, demonstrating the high quality of the sample material.

Supervised Hierarchical Cluster Analysis of DNA Methylation Values Separates Cryopreserved And Hope-Fixed Samples from Formalin-Fixed Tissues

To address the question whether lung tumor tissue and the corresponding normal tissue fixed according to the HOPE protocol is suitable for DNA methylation analysis using the BeadChip technology, DNA prepared from cryopreserved, HOPE-fixed, or formalin-fixed tissue samples from the same patient were applied to array-based DNA methylation analysis.

An ANOVA performed on the DNA methylation data obtained from all normal lung tissue samples ($n = 18$) clearly separated formalin-fixed samples from HOPE-fixed and cryopreserved samples (FDR < 0.05, $\sigma/\sigma_{max} > 0.2$; Figure 1). A total of 109 loci were found 'differentially methylated' by this approach (Supplementary Table 2). By applying the identical thresholds, the analogous analysis of tumor samples resulted in only seven differentially methylated loci, probably due to the higher interindividual heterogeneity of the tumor samples as compared with normal lung tissue.

DNA Methylation Data Obtained from HOPE-Fixed Tissue Highly Correlate with Data Obtained from Cryopreserved Tissue Samples

To exclude interindividual effects caused by differences in the individual DNA methylation patterns of the patients, we analyzed in the next step the data obtained from the six patients separately. An unsupervised hierarchical cluster analysis separated primarily the samples of each patient according to malignancy of the samples (ie, lung cancer and corresponding normal lung tissue), subsequently according to the fixation technique applied to the sample (Figure 2).

Interestingly, in all sample sets HOPE-fixed samples clustered together with cryopreserved samples and separately from the corresponding formalin-fixed samples. This is in line with the results of a correlation analysis demonstrating high correlation between cryo- and HOPE-preserved samples (normal

tissue: mean: 0.991, range: 0.989–0.994; tumor tissue: mean: 0.983, range: 0.971–0.993) and lower correlation between cryopreserved and formalin-fixed samples (normal tissue: mean: 0.975, range: 0.943–0.987; tumor tissue: mean: 0.960, range: 0.919–0.981, Supplementary Figure 2). Consequently, the DNA methylation patterns obtained from cryopreserved tissues samples were more similar to ones of HOPE-fixed samples than to the methylation patterns obtained from formalin-fixed tissues.

To exclude tissue or tumor heterogeneity as putative cause of these findings we performed DNA methylation analysis on three lung tumor cell lines (A549, H1650, and H838), which were fixed and processed according to the same protocol as the tissue samples. Both, PCA performed on the complete data set as well as hierarchical cluster analysis restricted to loci with high variance were found in line with results obtained from tissue samples (Supplementary Figure 3).

Formalin-Fixation Leads To an Increase in Variability of DNA Methylation Data As Compared to HOPE Fixation

As tumor heterogeneity might result into an increased variability when comparing differentially fixed tumor samples, we focused in the subsequent analyses on normal tissue samples.

First, to exclude loci with minor and probably biologically irrelevant variability in their methylation values from the analysis, we applied a variance threshold filter of $\sigma/\sigma_{max} > 0.3$ to the data. This approach identified up to 9149 highly variable DNA methylation values (mean: 2 490 loci; range: 175–9149 loci; Figure 3) in material obtained from the same patient, demonstrating the impact of the fixation technique
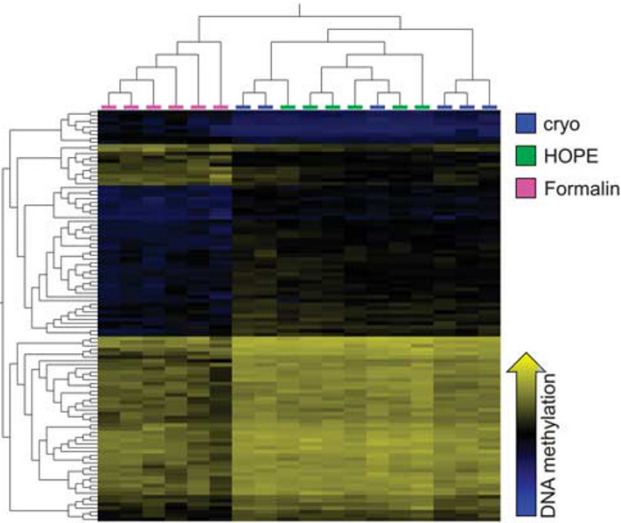
Figure 1 Hierarchical cluster analysis and heatmap of 109 CpG loci found differentially methylated in cryopreserved, HOPE-fixed, and/or formalin-fixed sample tissues as determined by an ANOVA (FDR < 0.05; $\sigma/\sigma_{max} > 0.2$; linkage: weighted average). Except cryopreserved tissues all samples were paraffinized. Only data obtained from normal lung tissue were included. Blue bars: cryopreserved samples, green bars: HOPE-fixed, and pink: formalin-fixed tissue samples. Heatmap: blue: low, yellow: high DNA methylation values.
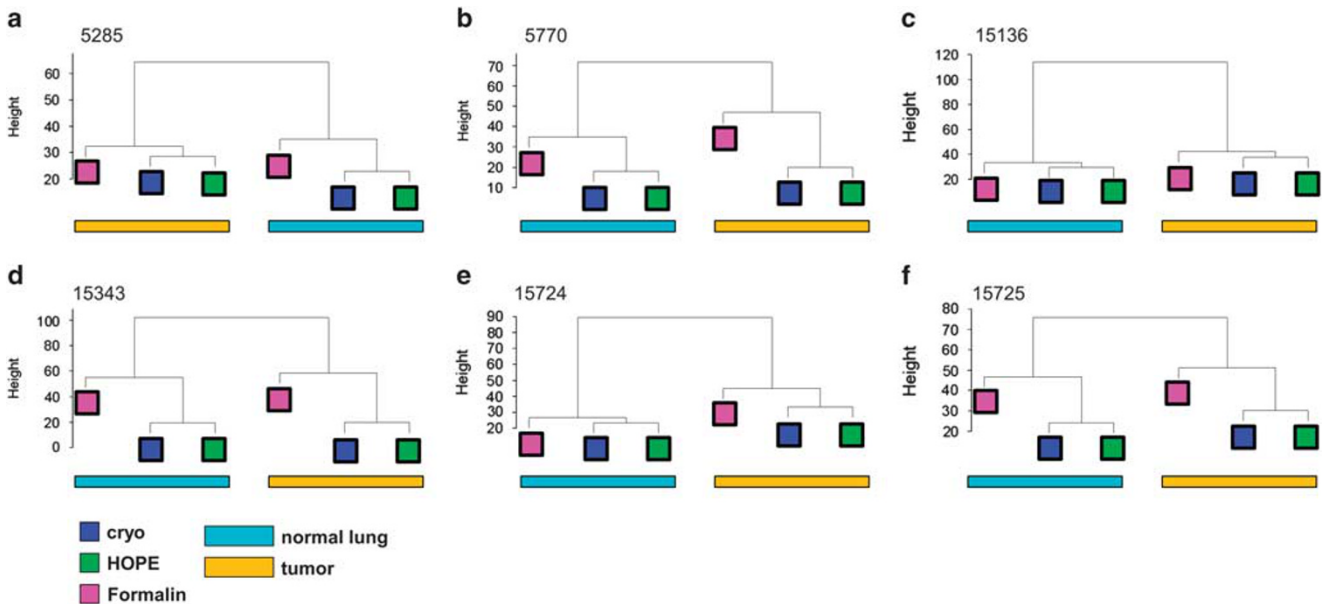
Figure 2 Hierarchical cluster analysis (euclidian distance) of DNA methylation values obtained from cryopreserved (blue squares), and HOPE- (green squares) or formalin-fixed samples (pink squares) of normal lung tissue (turquoise bar) and lung cancer tissue (orange bar). Samples from all patients (patient number is indicated on top) have been analyzed independently.
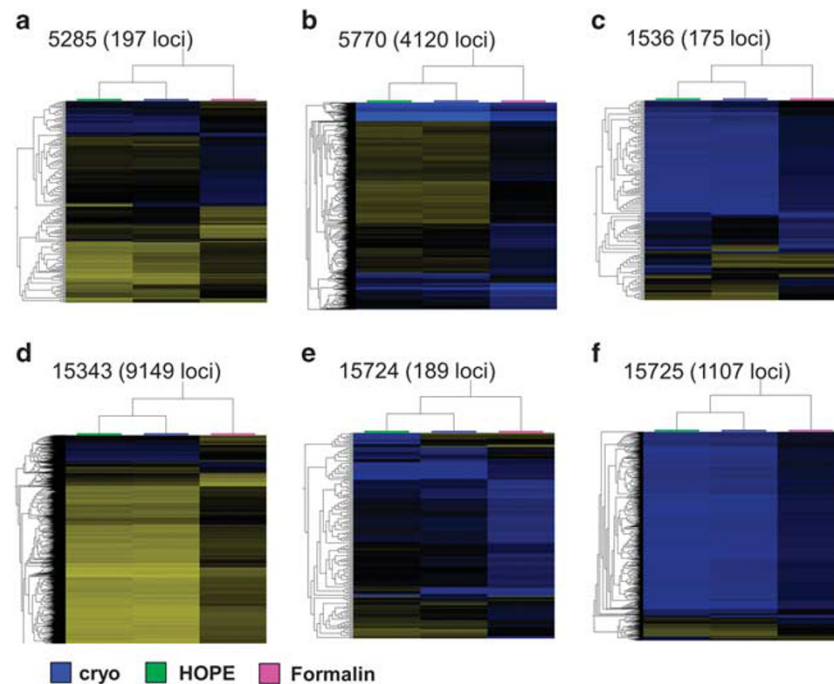
Figure 3 Unsupervised cluster analysis and heatmap of DNA methylation values obtained from the normal lung tissue of each individual patient. A filter of variance has been applied ($\sigma/\sigma_{max} > 0.3$; linkage: weighted average). The number of loci passing this threshold is indicated for each patient. Blue bars: cryopreserved samples, green bars: HOPE-fixed and pink: formalin-fixed tissue samples. Heatmap: blue: low, yellow: high DNA methylation values.

on the methylation data collected. Again, while the DNA methylation data obtained from the cryopreserved and the HOPE-fixed tissues were well comparable, the DNA methylation patterns obtained from the corresponding formalin-fixed tissues showed increased variability.

Finally, we investigated whether the variability in the methylation data found between differently preserved samples depends on the absolute methylation values. As shown in Figure 4a differences in the DNA methylation values of individual CpG loci between the cryopreserved normal lung samples and the corresponding HOPE-treated tissues usually were below 0.2 (sum of abs. differences = 9747.5, mean of absolute differences = 0.02, s.d. = 0.019; only 33 loci showed a difference > 0.2) and highest for intermediately methylated loci in cryopreserved samples. Remarkably, differences between cryopreserved samples and formalin-fixed samples were generally larger (sum of abs. differences = 25 861, mean of absolute differences = 0.05, s.d. = 0.056; 13 719 loci showed a difference > 0.2) and most prominent in the lowly and highly methylated fractions (Figure 4b).

We found differences between cryopreserved and HOPE-fixed samples on the one hand and formalin-fixed samples on the other hand in sample material collected from normal lung, lung carcinoma as well as lung cancer cell lines. Interestingly, none of these findings could be verified by bisulfite pyrosequencing (Supplementary Table 2), indicating that this is a technical issue related to the BeadChip processing protocol, rather than originating from biological differences due to cross-linking or nucleic acid denaturation caused by formalin.

DISCUSSION

Epigenetic is a major key player in the establishment of tissue and developmental-specific gene expression pattern.[16] Consequently, the DNA methylation is not only in the focus of numerous research projects but also entered the clinic.[23] The Infinium HumanMethylation450K BeadChip is an appropriate tool to get an overview about the DNA methylation pattern of tissue samples and widely used in numerous projects.[19,24] For many studies, FFPE material is a major source for DNA preparation. Although formalin itself does not influence the methylation of DNA,[25] widely available FFPE material is regarded as problematic for whole-genome amplification[21], which is a crucial part of the Illumina Infinium protocol. Indeed, altered DNA methylation determinations due to formalin-fixation causing unreliable DNA methylation values when applying the BeadChip technology are well known[18] and protocols to circumvent these problems have been developed.[26] However, these protocols need additional steps and are labor-intensive. Therefore, alternative sample fixation techniques which are compatible with the BeadArray technology and which produce fewer artifacts are of high interest.

In this study we compared DNA methylation values obtained by applying the BeadChip technology from cryopreserved, HOPE- and formalin-fixed tumor, and normal lung tissue
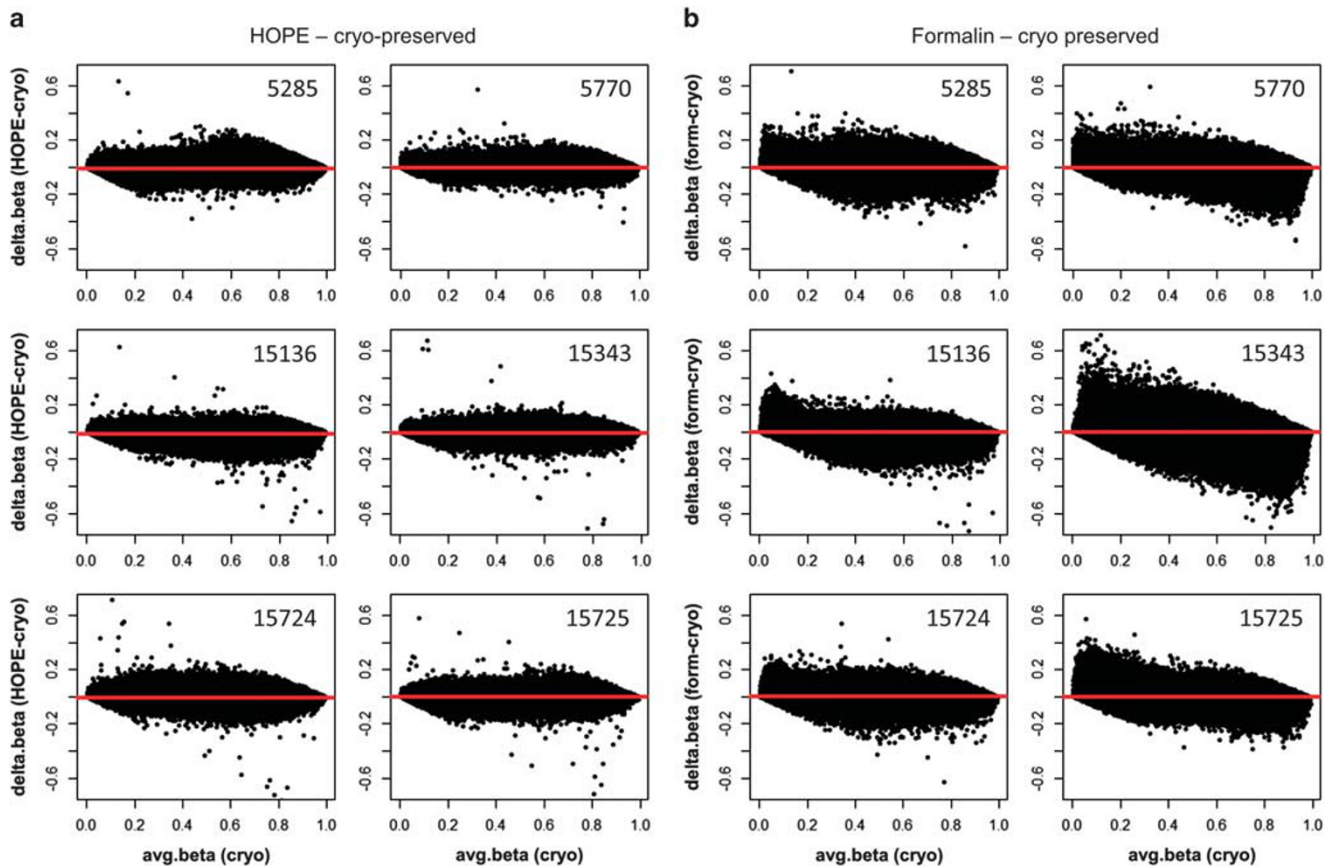
Figure 4 Scatterplot of DNA methylation data to demonstrate differences in the DNA methylation patterns of cryopreserved and HOPE-fixed tissues (**a**) or cryopreserved and formalin-fixed tissues (**b**), respectively. The DNA methylation values obtained from the cryopreserved sample are plotted to the *X* axis, the difference between the DNA methylation values obtained from the cryopreserved sample and the HOPE- or formalin-fixed samples are plotted to the *Y* axis. The red line indicates no difference. The number indicates the patient providing the sample material.

samples of six patients suffering from lung carcinoma as well as three widely used lung cancer cell lines. In general, good reproducibility of the array and good correlation with results obtained by methylation-specific PCR (MSP), bisulphite pyrosequencing, and bisulphite sequencing has been shown before by us and others.[27,28]

Comparing the DNA methylomes of normal lung tissues from six patients each preserved by three techniques, we identified 109 CpG loci commonly differentially methylated (FDR < 0.05, $\sigma/\sigma_{max} > 0.2$) in formalin-fixed samples as compared with the other samples. In contrast, DNA methylation values obtained from HOPE-fixed and cryopreserved samples were highly comparable. This remained true, when performing unsupervised hierarchical cluster analyses either on the complete data sets or when applying a filter of variance ($\sigma/\sigma_{max} > 0.3$) to focus on loci with high variance in their DNA methylation pattern. As these results can be verified in cell lines, tissue or tumor heterogeneity can be excluded as a cause for our findings. Interestingly, while HOPE-fixed and cryopreserved tissue samples differed mainly in the partially methylated loci, formalin-fixed samples differed in particular in the lowly and highly methylated loci.

Our data support previous reports that formalin-fixation interferes with a subsequent DNA methylation analysis using the BeadChip technology.[26] Furthermore we showed that HOPE fixation is superior to formalin-fixation if DNA methylation analysis using this technology is intended. This demonstrates that the fixation method applied is a serious confounder variable for BeadChip DNA methylation analysis that has to be carefully considered. Even although the cryopreserved samples which served as standard material for the comparisons were not micro-dissected as the formalin- or HOPE-fixed samples, HOPE-fixed samples displayed higher similarity to the cryopreserved tissues. Hence, the enrichment for tumor DNA for analysis displays a smaller effect on the outcome of the analysis as the applied fixation technique. Furthermore, artifacts related to formalin fixation were restricted to BeadChip analysis and could not be verified by bisulfite pyrosequencing, excluding a chemical reaction effecting the DNA methylation itself. Instead, our findings are in line with previous studies reporting technical problem with formalin-fixed samples when using the BeadChip technology.[18,26]

The HOPE technique[10] is a preservation technique that leads to paraffin-embedded tissues without cross-linking

of proteins[13] and only little degradation of nucleic acids.[11] Recently it has been shown that HOPE-fixed biomaterial allows a multitude of different molecular approaches such as 2D electrophoresis[7] or transcriptome analysis[29] out of the very same paraffin block. As the tissue processing takes 1 day longer than classical FFPE material, this might raise objections for application in routine diagnostics work-flows and therefore favors its use for biobanking or research-orientated approaches. Thus, it is a highly valuable source for a holistic read-out from biobank material on all biological levels including excellent tissue morphology. With regard to small or rare samples such as biopsies or and a growing number of desired read-out techniques from the same specimen, the choice of preservation is crucial for later-on investigations and a possible pitfall in biobanking approaches. HOPE fixation therefore, might be a reliable alternative for formalin-fixation when molecular investigations, eg, array-based DNA methylation analysis, are intended to apply. In comparison to cryopreserved specimen as the gold standard for molecular analysis, HOPE-preserved specimen retain an excellent morphology and do not show severe degradation of nucleic acid integrity over time. Therefore, it delivers the same histologic information as FFPE material but retains the nucleic acid integrity and does not cross-link antigens, which facilitates antibody-based approaches.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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