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Molecular Fixative Enables Expression Microarray Analysis of Microdissected Clinical Cervical Specimens

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Abstract

Formalin-fixed tissue has been a mainstay of clinical pathology laboratories, but formalin alters many biomolecules, including nucleic acids and proteins. Meanwhile, frozen tissues contain better-preserved biomolecules, but tissue morphology is affected, limiting their diagnostic utility. Molecular fixatives promise to bridge this gap by simultaneously preserving morphology and biomolecules, enabling clinical diagnosis and molecular analyses on the same specimen. While previous reports have broadly evaluated the use of molecular fixative in various human tissues, we

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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present here the first detailed assessment of the applicability of molecular fixative to both routine histopathological diagnosis and molecular analysis of cervical tissues. Ten specimens excised via the Loop Electrosurgical Excision Procedure, which removes conical tissue samples from the cervix, were cut into alternating pieces preserved in either formalin or molecular fixative. Cervical specimens preserved in molecular fixative were easily interpretable, despite featuring more eosinophilic cytoplasm and more recognizable chromatin texture than formalin-fixed specimens. Immunohistochemical staining patterns of p16 and Ki-67 were similar between fixatives, although Ki-67 staining was stronger in the molecular fixative specimens. The RNA of molecular fixative specimens from seven cases representing various dysplasia grades was assessed for utility in expression microarray analysis. Cluster analysis and scatter plots of duplicate samples suggest that data of sufficient quality can be obtained from as little as 50 ng of RNA from molecular fixative samples. Taken together, our results show that molecular fixative may be a more versatile substitute for formalin, simultaneously preserving tissue morphology for clinical diagnosis and biomolecules for immunohistochemistry and gene expression analysis.

Keywords

cervical intraepithelial neoplasia; gene expression microarray analysis; immunohistochemistry; microdissection; molecular fixative

Introduction

Advances in molecular biology have greatly improved our understanding of biological systems. Despite the wealth of cell lines, animal models, and other model systems, there remains no true substitute for clinical specimens to probe the molecular mechanisms underpinning human health and disease.

Once collected, specimens must be fixed to preserve them in a state as close to their native state as possible and to prevent tissue response to excision and further decay. Two commonly used fixation techniques in clinical settings are immersion in formalin solution and freezing. Formalin fixation is considered the gold standard for clinical diagnosis. Formalin fixation preserves the tissue and cellular morphology in a fashion that pathologists rely upon to make their diagnoses, while maintaining the immunoreactivity of many antigens. Consequently, there is increasing interest in extracting biomolecules from archival formalin-fixed paraffin embedded (FFPE) samples in clinical laboratories around the world for molecular studies. However, chemical alterations to proteins, DNA, and especially RNA caused by formalin have been well-documented (Lehmann and Kreipe, 2001; Lewis et al., 2001; von Ahlhen et al., 2007; Werner et al., 2000), including cross-linking of proteins and fragmentation and covalent modifications of nucleic acids (Dotti et al., 2010; Masuda et al., 1999). In contrast, freezing specimens preserves biomolecules better, but affects cellular morphology, limiting the use of frozen specimens for diagnostic purposes (Vincek et al., 2003). Moreover, freezing must be done immediately, as any delay will result in biochemical changes in the tissue. In order to align molecular analyses with clinical diagnoses, researchers have typically collected adjacent specimens, fixing one in formalin and freezing the other.

The observation that a high-concentration aqueous sulphate solution precipitated out RNases at room temperature led to the development of *RNAlater* (originally marketed by Ambion, now part of Life Technologies, Carlsbad, CA, USA) (Florell et al., 2001; Vincek et al., 2003). *RNAlater* was found to effectively preserve RNA, allowing samples to be processed at a different place and time from collection (Florell et al., 2001; Mutter et al., 2004). Unfortunately, *RNAlater* alone resulted in uneven immunohistochemical staining and preserved noticeably less of the finer structural details compared to formalin (Paska et al., 2004). This could be improved by post-fixing with formalin, but formalin is known to damage RNA (Gugic et al., 2007; Masuda et al., 1999).

Alcohol-based molecular fixatives have been introduced that aim to combine the best attributes of formalin and freezing (Delfour et al., 2006; Ergin et al., 2010; Stanta et al., 2006; van Hemel and Suurmeijer, 2013; Viertler et al., 2012; Vincek et al., 2003). Samples are processed in a manner similar to formalin, including embedding in paraffin and subsequent sectioning to glass slides. However, preservation of biomolecules is decidedly superior to that of formalin (Nassiri et al., 2008; Turashvili et al., 2012). Moreover, clinical diagnosis and molecular analysis can now be performed from the same sample block. While molecular fixatives have been tested on a number of human tissue types (Turashvili et al., 2012; Vincek et al., 2003), little is known about their effect on cervical tissue. Successes reported for other tissue types suggest that molecular fixative will preserve biomolecules while maintaining morphological features necessary for clinical diagnosis in cervical specimens. The improved preservation of biomolecules should enable the use of molecular fixative preserved paraffin-embedded (MFPE) cervical samples for microdissection and subsequent microarray analysis.

The present evaluation of molecular fixative is part of a larger effort to uncover novel biomarkers for early detection of cervical cancer through the microdissection of epithelial layers (Li, 2012). Normal human cervical squamous epithelium consists of a differentiating continuum of cell layers. It is hypothesized that the basal layer consists of stem cells, with cells maturing and differentiating as they migrate towards the surface. Carcinogenesis is a long, multi-step process that upsets this regulated program of cell maturation. By studying differences in expression between cervical epithelial layers across various grades of cervical intraepithelial neoplasia (CIN), we seek to explore the molecular basis of the early carcinogenic process.

While many approaches to studying genome-wide expression in cervical cancer have been used in the past, including serial analysis of gene expression (Kneller et al., 2007; Shadoo et al., 2008; Shadoo et al., 2007), the present study employs oligonucleotide microarrays (Trachtenberg et al., 2012). However, microarray studies have typically compared invasive cancer to normal controls (Ahn et al., 2004; Manavi et al., 2007; Shim et al., 1998; Sun et al., 2007; Wilting et al., 2008; Wong et al., 2006), ignoring any changes that might be occurring during the carcinogenic process through the various grades of dysplasia. Even when CIN is studied, such studies tend to treat the entire epithelium as one homogeneous whole (Chen et al., 2003; Gius et al., 2007; Hudelist et al., 2005; Kendrick et al., 2007; Sopov et al., 2004; Zhai et al., 2007), ignoring subtle differences in expression between epithelial layers that might be playing a crucial role in carcinogenesis and that do play a

significant role in the pathological identification of the different grades of CIN. Microarray analysis of CIN epithelium microdissected into separate layers, then, might offer a novel approach to understanding the early genetic changes underpinning carcinogenesis in cervical squamous epithelium.

Previous microarray studies of CIN have tended to use frozen samples (Ahn et al., 2004; Chen et al., 2003; Gius et al., 2007; Wilting et al., 2008; Wong et al., 2006; Zhai et al., 2007), using adjacent blocks to infer the diagnosis of the assayed block. Molecular fixative promises to enable histological assessment and molecular analysis on the same block, allowing for more precise correlations between the molecular data and histology. Used in this way, histology can directly guide microdissection of the sample to be used for microarray analysis. However, little is known about the use of molecular fixative in cervical specimens. Hence, in this work, we assess the suitability of using molecular fixative preserved cervical specimens for simultaneous clinical diagnosis and gene expression microarray analysis of small quantities of RNA isolated from microdissected samples.

Materials and Methods

Samples

To assess the preservation of tissue architecture and suitability for clinical diagnostic use, tissue excised from 10 patients who had undergone loop electrosurgical excision procedure (LEEP) at the British Columbia Cancer Agency (BCCA) was cut radially into pieces, with alternating pieces being preserved in formalin or Tissue-Tek® Xpress® Molecular Fixative (Sakura Finetek, Torrance, CA, USA), a proprietary, methanol-based molecular fixative formulation. This was done within an hour of the tissue being removed from the patient and each LEEP was cut so as to generate the same number of formalin blocks as would have resulted from following standard protocol (typically 6 or 7 per case). Following overnight fixation, formalin-fixed samples were processed and embedded in paraffin according to standard clinical protocols at the BCCA, while molecular fixative samples were processed and embedded in paraffin on the Tissue-Tek® Xpress® Rapid Tissue Processor (Somagen Diagnostics, Edmonton, AB, Canada) according to the manufacturer's extended protocol by the Centre for Translational and Applied Genomics at the BCCA.

For microarray analysis, seven biopsies from patients about to undergo LEEP were excised and rapidly (all within 15 minutes, typically within 5 minutes) fixed in Tissue-Tek® Xpress® Molecular Fixative (Sakura Finetek) and embedded in paraffin (MFPE). Except where otherwise noted, all references to molecular fixative and MFPE refer to this one from Sakura Finetek and not other competing products or *RNAlater*.

All samples were collected primarily from patients with CIN II or CIN III, but the actual regions collected had a range of histopathological grades ranging from normal to CIN III. In this study, all regions with histopathological grades of CIN II or worse will be considered high-grade. Approval was granted by the Research Ethics Boards of the BC Cancer Agency and The University of British Columbia. Written informed consent was obtained from all participants.

Sample preparation

Molecular fixative samples were handled like routine formalin-fixed paraffin-embedded blocks and all samples were generally handled according to standard clinical protocols. LEEP blocks for assessing diagnostic suitability were sectioned at 4 μm . For formalin blocks, sections were taken at 3 levels: near the surface, near the middle, and roughly halfway between the first two. At each level, one section was stained with hematoxylin and eosin (H&E) while at least two additional serial sections were cut and saved for future analyses, including immunohistochemistry as documented below. MFPE blocks were sectioned at only one level (near the surface), with 4 additional serial sections cut and saved per block.

MFPE biopsy blocks for microdissection were cut at 8 μm , 4 sections per slide, and deparaffinized before use. 100 sections per block were cut, with reference slides cut about every 20 sections and stained with H&E.

Histological grading

The hematoxylin and eosin-stained slides were scanned using a whole slide imager (Pannoramic MIDI, 3DHistech, Budapest, Hungary). Using the high-resolution digital images produced by the scanner, the study pathologist (DvN) graded and circled all regions of abnormality. This information was used to assess clinical suitability and to guide microdissection of the adjacent unstained sections.

Immunohistochemistry

Immunohistochemical staining of p16 and Ki-67 were simultaneously assessed by using the CINtec® PLUS double staining kit (Roche mtm laboratories AG, Heidelberg, Germany), following the manufacturer's instructions. One slide from each block of each of the 10 alternately fixed LEEP cases was stained, digitized, and interpreted by the study pathologist.

Microdissection

Manual microdissection was performed at room temperature using a needle and a dissecting microscope. The dewaxed MFPE biopsy slides were kept on dry ice until just before microdissection. The collected sample was transferred to a tube of Buffer PKD (this is a component of the QIAGEN RNeasy FFPE Kit used for RNA purification, see below). Epithelium was collected as two layers: top (superficial) half and bottom (basal) half. In addition, the stroma immediately underlying (roughly 200–500 μm) the collected epithelium was selectively scraped off as a separate layer. Different needles were used for each layer of each sample.

RNA extraction and purification

RNA from MFPE biopsy samples was purified using the QIAGEN RNeasy FFPE Kit (Toronto, ON, Canada), following the manufacturer's instructions. RNA amount and purity were assessed using NanoDrop (Thermo Scientific, Wilmington, DE, USA), while degradation was assessed by 0.8% agarose gel electrophoresis assay of the sample with the highest quantity of RNA.

Microarrays

RNA was amplified and labelled using the Agilent Low Input Quick Amp Labeling kit (Agilent Technologies Canada, Mississauga, ON, Canada). Labelled cRNA yield and quality were assessed according to the instructions provided by Agilent. Expression analysis on successfully labelled samples was performed using the Agilent Whole Human Gene Expression Microarray Kit, 4×44K, following manufacturer's protocol. These arrays assay over 41000 unique probes spanning the human genome. Each array slide allows up to 4 samples to be assayed simultaneously. In some instances, the same sample was run twice (e.g., with different RNA input amounts into the labelling reaction) to serve as an indicator of the reproducibility of the data. The hybridized microarray slides were scanned using a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA) running GenePix Pro version 6.1 software.

Microarray data analysis

Data manipulation was performed in Microsoft Office Excel (Redmond, WA, USA) for spreadsheet functions and STATISTICA (StatSoft Inc., Tulsa, OK, USA) for statistical analysis.

The microarray data (background-subtracted intensity values) were normalized for each array by dividing by the median intensity value of the spots on the array. Zero and negative values were deleted and data from probes with multiple spots on the array were consolidated by removing the highest and lowest intensity values and averaging. From this, a set of normalized intensity values for each unique probe was obtained. Some genes were represented by multiple probes, but these data were not averaged. The resulting data set consisted of 41000 intensity values per sample, one for each unique probe on the microarray.

For each sample, modified M-A plots (Quackenbush, 2002; Smyth and Speed, 2003) were generated by plotting $M = \log_2(T/B)$ against $A = \log_2(T \cdot B)$, where T and B are the array-median normalized intensity values for the top and bottom layers, respectively. Assuming that the majority of probes are expressed at similar levels between the layers, a further adjustment to the data can be made to set the central log-ratio M of each plot to be zero. Using all data with $A > 4$ to avoid fitting to data that is excessively noisy due to a weak signal, a linear regression for each M-A plot was calculated and subtracted from all data in that plot.

Samples that were run in duplicate were used to determine technical variability. Instead of comparing top against bottom layers, M-A plots were constructed by comparing the duplicate samples. One approach to identify differentially expressed genes is to use the regions of technical variability generated by M-A plots of duplicate samples as thresholds of significance with which to analyze the top versus bottom data of the other samples. This has the advantage of being simple to perform while making minimal distribution assumptions (e.g., normally distributed or not) in terms of differentially expressed transcripts. To this end, after linear adjustment as above, double exponential functions were fit manually in an attempt to replicate the envelopes traced by the duplicate data M-A plots. Once overlaid on

the top versus bottom data, these fits defined the range of expected technical variability. As the range and scaling of microarray data are somewhat arbitrary, the fits were translated so that the maximum (saturation) A values of the duplicate data underlying the fits and the test data were aligned. Measurement data points lying outside these fits could then be considered as having potential differential expression. To estimate the false discovery rate of using these fits, this method was applied to the duplicate data, where there is no biological variation between the samples. The focus of the present paper is to assess the technical validity of the approach, in order to gauge the suitability of using MFPE specimens for gene expression microarray analysis. The selection and validation of candidate biomarkers from the data set will be the subject of a future manuscript.

To further evaluate the quality of the microarray data, unsupervised hierarchical cluster analysis was performed on all log-transformed data collected from arrays run with an input of at least 50 ng RNA to start the labelling reaction. All saturated and low-intensity (<16 or $\log_2 < 4$) data were removed from this analysis. Complete linkage was required between grouped clusters. We used the Pearson distance metric, which is more likely to capture overall correlations within the data and is less sensitive to imperfect between-array normalization than metrics like Euclidean distance (Eisen et al., 1998).

Results

Histology and immunohistochemistry comparison

Compared to formalin-fixed samples, H&E slides of MFPE samples were detectably different but nevertheless quite easily interpretable. MFPE cell nuclei chromatin had a different appearance, with more recognizable texture. Nucleoli were more prominent and cytoplasm was more eosinophilic in the MFPE samples (Figure 1).

Interpreting all LEEP blocks available for each patient, there was good agreement between fixatives when it came to identifying the highest grade of CIN in the patient, with all cases being at most one grade apart. Six out of ten cases matched exactly in terms of the highest grade of CIN found. If CIN II and worse is considered a high-grade lesion, then the two fixatives agreed on identifying high-grade lesions in 9 out of 10 cases. Given that the same lesions may not always span multiple blocks, this level of disagreement was expected.

Immunohistochemical staining of p16 and Ki-67 was comparable between fixatives, with regions of confluent positive p16 staining for both fixatives aligning well with regions of CIN identified from the H&E slides (Figure 2). Ki-67 staining in molecular fixative generally appeared to be more sensitive, resulting in more positive cells than in the formalin samples. This is particularly noticeable in regions of benign histology, where a high proportion of parabasal and basal cells are intensely stained in MFPE while the same cells are only weakly stained in FFPE (Figure 3). Nevertheless, in all 10 LEEP cases, we found agreement between the fixatives on identifying high-grade (CIN II or worse) cases. All high-grade lesions exhibited a combination of confluent positive p16 staining and positive Ki-67 staining in the superficial layer of the squamous epithelium. This suggests that the antigenicity of both cytoplasmic (p16) and nuclear (Ki-67) antigens can be preserved by molecular fixative.

All in all, despite differences in cellular appearance and staining intensity, compensating for the observed differences was found to be straightforward and our study pathologist felt comfortable using MFPE slides to make clinical diagnoses.

Manual microdissection

Manual microdissection was tedious, but largely successful and feasible. Upon moving slides from dry ice to the room temperature dissecting microscope, a small amount of moisture condensed on sections. This was found to aid in microdissection by binding the collected tissue to the needle as it was being scraped, whereas dry tissue was easily propelled great distances (and hence lost) by the reflex action of the needle as the tissue detached from the slide. Moreover, the slightly moist epithelium was found to hold together well and separate from the stroma cleanly at the basement membrane when gently pulled by the needle. Separating the top and bottom layers of the epithelium was best done by scoring completely dry sections with a needle point.

Preservation of RNA

Nine regions from the MFPE biopsy blocks of 7 patients were microdissected, including 1 normal, 3 CIN I, 3 CIN II, and 2 CIN III regions. Two patients had regions of both CIN II and CIN III, which were collected separately and labelled as A and B. Working on multiple slides at once allowed microdissection to proceed more quickly, allowing 12 sections to be completed in about 30 minutes. An example of one sample undergoing the various stages of microdissection is shown in Figure 4. Photos documenting microdissection of all the samples can be found in the Supplementary Data.

RNA was successfully purified from all manually microdissected MFPE biopsy cases. Most of these were successfully assayed on the gene expression microarrays. For one CIN I case (case 0027), the stroma was microdissected but RNA has not yet been purified. For a CIN II case (0053A), the stroma RNA was purified, but yield and quality were insufficient and the labelling reaction failed. A gel comparing RNA from MFPE and FFPE samples showed significantly more degradation in the FFPE sample (Figure 5). As mRNA makes up a small fraction of total RNA and is normally not directly visible on a gel, the rRNA bands are typically used as a proxy for determining RNA integrity. In the MFPE sample, the 18S band appeared distinctly, while the 28S band was less well-defined. Despite some degradation of the 28S band, we decided to press ahead with microarray analysis on MFPE samples based on prior success with samples exhibiting a similar gel pattern. The FFPE sample, however, was deemed to be too degraded to pursue gene expression microarray analysis. Hence, only MFPE samples will be used in the subsequent microarray experiments.

Three samples were assayed on the microarrays in duplicate: A normal (case 0043) bottom layer run with 200 ng and 50 ng input into the labelling reaction, a CIN I (0044) top layer run with two 200 ng aliquots as input, and a CIN III (0028) bottom layer run with 200 ng and 25 ng as input. The first two sets of duplicates showed an excellent linear correlation (e.g., Figure 6), but the third set exhibited a significant side branch (Figure 7), i.e., there was a cluster of data whose behaviour noticeably deviated from the trendline established by the main set of data.

It appears that reproducible microarray data can be obtained using 50–200 ng RNA as input to the labelling reaction. In the M-A plots, the normal data produced slightly more scatter than the CIN I data (Figure 8), so their fits will be referred to as the wide and narrow thresholds, respectively.

False discovery rates

The accuracy of the fitting can be assessed by counting the number of false positives that the fit would “discover” in the duplicate data from which it was derived. Each set of thresholds was overlaid over each set of duplicate data and the number of data points outside the thresholds was determined. Additionally, as a narrow threshold would result in a high false discovery rate on its own, additional criteria of a greater than two-fold change ($|M| > 1$) and a sufficiently high intensity ($A > (\max A) - 20$) were applied. The results are shown in Table 1. Note that the intent here is to quantify the expected false discovery rate of this approach attributable to technical scatter in the data and that the identification and validation of specific differentially expressed genes will be the subject of a future manuscript.

With 5 high-grade (CIN II or worse) and 4 low-grade (CIN I) or normal samples, we could require that for a change to be considered significant, it needed to occur in at least 3 samples of the same group (high- or low-grade) and in the same direction. Taking the most permissive scenario, narrow thresholds on widely scattering data, this yields a probability of $2.5 \times 10^{-5} \%$ that a probe would register as a false positive for at least 3 out of 5 samples, or about 0.01 probes in a microarray of 41000. This number drops further if we add the intensity and fold-change criteria or we add more samples as we continue this study. Hence, even the narrow thresholds will yield a small number of false positives attributable to technical variability if we require that multiple samples must agree.

Cluster analysis

Cluster analysis revealed that replicate samples and epithelial samples from the same patient tended to cluster together (Figure 9). The two data sets corresponding to the same hybridized array scanned twice with different gain settings (0044 TopB) were the most similar, followed by a replicate array of the same sample (0044 TopA). The stroma samples formed their own clusters. For case 0053, the CIN II and III regions were identified in essentially the same region on different H&E slides. Hence, the A and B samples constitute adjacent regions that differ only by their depth in the tissue block. As high-grade lesions, the top and bottom layers also appear morphologically similar. We observe, then, that all four epithelial samples from case 0053 cluster closely together. A similar situation is observed with case 0033 and again, its four epithelial samples cluster together. Of the 7 patients studied, 6 clustered largely as expected. However, case 0028 was somewhat anomalous as its three layers clustered separately from the others. It was the first MFPE sample processed and our relative inexperience with the procedures at the time may have played a role.

Discussion

Formalin fixation has routinely been used in laboratories all around the world for processing human specimens for clinical use. However, the deleterious effects of formalin fixation on

biomolecules have been well documented and present a significant challenge to attempts to use such material for molecular biological studies (Lehmann and Kreipe, 2001; Lewis et al., 2001; von Ahlhen et al., 2007; Werner et al., 2000). Frozen samples, on the other hand, produce better quality biomolecules (Penland et al., 2007; Turashvili et al., 2012), but at the cost of being unreliable for clinical use. Molecular fixative has been proposed as a fixative that will allow clinical diagnosis and molecular biology to be performed on the same sample (Vincek et al., 2003). Molecular fixative has already been shown to preserve morphology of tissues nearly as well as formalin in some tissues (Turashvili et al., 2012; Vincek et al., 2003). RCL2 and PAXgene, commercially available alcohol-based fixatives that compete with the brand of molecular fixative used here, were recently shown to perform well on a number of tissue types, including cervical specimens (Masir et al., 2012; Staff et al., 2013). However, comparative studies have suggested that not all molecular fixatives perform equally well (Staff et al., 2013). Previous studies have tested Sakura Finetek's Tissue-Tek® Xpress® Molecular Fixative (the one used in the present study) on a variety of human tissues (Kashofer et al., 2013; Morales et al., 2004; Nadji et al., 2005). As part of a study involving many tissue types, Morales *et al* (2008) reported that material prepared in molecular fixative, including cervical tissue, was comparable to formalin-fixed specimens in terms of tissue architecture, while yielding stronger immunohistochemical staining and better preservation of biomolecules for molecular assays. However, no details were provided and, in fact, our results indicate that there are distinct and visible differences between formalin and molecular fixative preserved tissue sections stained with H&E or immunohistochemistry. To our knowledge, this paper represents the first in-depth study on the suitability and effect of the Sakura Finetek molecular fixative for histopathological and molecular analysis of cervical tissue.

In order for a molecular fixative to be a suitable replacement for formalin in a clinical laboratory, MFPE specimens must at a minimum be useful for the tasks currently considered the domain of FFPE specimens, namely preserving the tissue architecture that pathologists rely upon to make diagnoses and proteins for immunohistochemical analysis. Cytoplasm in MFPE specimens were found to be more eosinophilic, so H&E staining protocols could be modified to increase hematoxylin staining times while reducing eosin staining times. This was found to make the relative stain intensities more similar to formalin-fixed slides, but was not done routinely in the present study. H&E-stained MFPE slides were visibly different from their FFPE counterparts, with more pronounced chromatin texture features, but such differences were found not to alter most of the primary characteristics used in grading, such as nuclear hyperchromasia, mitotic activity, and nuclear cytoplasmic ratio. Indeed, when comparing the highest grade lesion found in alternating formalin and molecular fixative blocks of the same LEEP, the results were always within one grade of each other. On a patient-by-patient basis, there was almost complete agreement between the fixatives in identifying high-grade cases. Histological interpretation is by its nature subjective. Moreover, it is possible that the highest grade lesion did not span both a formalin block and a molecular fixative block.

In addition to histology, cervical pathologists are also particularly interested in immunohistochemical staining of p16 and Ki-67. In our hands, dual staining of both markers

using the CINtec kit was comparable with both fixatives. Grading of the CINtec-stained LEEP cases was nearly perfectly correlated between fixatives while closely mirroring the H&E grading results. As noted by other groups (Morales et al., 2004; Nadji et al., 2005), staining conditions may need refinement to optimize staining, but this is generally within the scope of what all labs deal with when working with new antibodies. We did, however, observe stronger staining of Ki-67 in MFPE specimens, a result that was also seen in standalone immunohistochemical staining of Ki-67 with the more commonly used monoclonal antibody MIB-1 (Dako Canada, Mississauga, ON, Canada). Increased sensitivity of the Ki-67 staining might be compensated for during interpretation of staining, similar to how the more pronounced textures observed in the H&E slides can be handled, or staining protocols may be modified to reduce staining by increasing dilution of antibodies, reducing staining time, or reducing antigen retrieval time, for example. Interestingly, our observation of increased sensitivity of Ki-67 staining with MFPE is the exact opposite of what the University of Miami group observed (Morales et al., 2004; Nadji et al., 2005). One of their studies used a different monoclonal antibody than the one we used, which might help to partly explain the discrepancy. This underscores the well-established principle that all laboratories should optimize their own immunohistochemical staining protocols for all antibody-fixative combinations.

However, to truly replace formalin, molecular fixative must provide something that formalin does not. To this end, we performed gene expression microarray analysis of microdissected biopsy samples, to test the ability of molecular fixative to preserve RNA. Gel electrophoresis showed that RNA from FFPE samples was significantly more degraded than from MFPE samples, so we decided not to pursue microarray analysis of the FFPE samples. Meanwhile, replicate and aliquot analysis as well as cluster analysis was used to get an impression of the reliability of the MFPE microarray data by looking at the general relationships between the samples. For cluster analysis, we chose to use the Pearson distance metric as it captures the correlations between genes via the Pearson correlation coefficient (Eisen et al., 1998). Relative expression levels for the majority of genes are expected to be similar in all the samples. The Euclidean distance, another common metric for cluster analysis, depends on the absolute expression differences between samples, leaving it sensitive to imperfections in between-array normalization. Cluster analysis was found to be sensitive to saturated data, but was sensitive to low-intensity data only when performed on log-transformed data. Hence, both saturated and low-intensity data were removed from the cluster analysis. There was little difference between the clustering results performed on log-transformed and untransformed data.

We would expect replicate samples to cluster most tightly together. Samples taken from the same patient would also be expected to cluster together as would perhaps samples of the same CIN grade. On the other hand, we would expect epithelial samples to cluster separately from stromal samples and, ideally, high-grade samples would cluster separately from normal and CIN I samples. In our data, we found that replicate and adjacent samples tended to cluster together and stroma generally clustered separately from epithelium. Most of the data clustered as expected and give us additional confidence in our data. Of note, samples from the same patient tended to cluster together, supporting the concept that there is significant

systematic biological variation in expression between patients, even those harbouring lesions of the same CIN grade.

All pair-wise comparisons of microarray data showed similar levels of expression between samples for the majority of probes. In MFPE samples, this was valid down to 50 ng of purified total RNA. The MFPE samples varied somewhat in the amount of scatter present, showing that there is some variability between different runs or samples in terms of the level of scatter one can expect. Duplicate samples, in particular, allow us to quantify the level of technical variability in the data (Table 1). In analyzing the data to uncover differentially expressed genes, one could further reduce the anticipated false discovery rate attributable to technical variability by requiring a minimum number of samples that must exhibit the same pattern before it is considered significant. Hence, our data suggest that molecular fixative adequately preserves RNA for microarray analysis of microdissected specimens.

Conclusions

The use of MFPE specimens could potentially revolutionize how clinical samples are handled due to the immense opportunity afforded by the ability to perform molecular studies on the same sample as that used for clinical diagnosis. Using molecular fixative, we could use our reference H&E sections to directly guide microdissection of the precise regions of CIN present in our specimens. The prevailing practice of using adjacent blocks clinical diagnosis and molecular analysis is very approximate at best. At worst, we have already observed cases where the lesions present at one end of the tissue block are not present at the other end, making it entirely possible that an adjacent block contains none of the lesions present in an examined diagnostic block. Our results demonstrate that MFPE specimens provide a reasonable substitute for formalin-fixed specimens, preserving sufficient tissue architecture to enable clinical histopathological diagnosis while preserving biomolecules for immunohistochemistry and gene expression microarray analyses.

Description of Supplementary Data

Microdissection of Molecular Fixative Samples

This document consists of a series of composite figures documenting the microdissection of each of the molecular fixative samples used in this study. Each figure is composed of five panels: A reference H&E section (**A**) is shown to the left beside photographs of an adjacent section before microdissection (**B**), after removing the top layer (**C**), after removing the bottom layer (**D**), and after removing the stroma (**E**). Microdissection was always performed in this order.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCCA	British Columbia Cancer Agency
CIN	cervical intraepithelial neoplasia
FFPE	formalin-fixed paraffin embedded
H&E	hematoxylin and eosin
LEEP	loop electrosurgical excision procedure
MFPE	molecular fixative preserved paraffin embedded

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Highlights

- We evaluated molecular fixative as a substitute for formalin in cervical tissues.
- Molecular fixative preserved morphology for clinical interpretation.
- Molecular fixative preserved immunohistochemical staining of p16 and Ki-67.
- Molecular fixative tissues were suitable for gene expression microarray analysis.

Figure 1.

Comparison between formalin (**A, C**) and molecular fixative (**B, D**) for H&E staining. The regions shown are benign (**A, B**) and CIN II (**C, D**) from case 0072. Scale bars are 20 μ m. To get higher magnification than with the whole slide imager, these images were acquired using a QImaging MicroPublisher (Surrey, BC, Canada) camera mounted atop a regular brightfield transmission light microscope with a 60x immersion oil objective lens.

Figure 2.

Comparison between formalin (A, B) and molecular fixative (C, D) for H&E (A, C) and CINtec immunohistochemical (B, D; brown = p16, red = Ki-67) staining in regions of CIN II in case 0072. Scale bars are 500 µm.

Figure 3. Comparison between formalin (A) and molecular fixative (B) for Ki-67 immunohistochemistry. The regions shown are benign from case 0072. IHC was performed using the CINTec kit as described in the text (red = Ki-67, brown = p16). Scale bars are 100 µm.

Figure 4.

Example composite figure documenting microdissection of case 0028. A reference H&E section (A) is shown alongside photographs of an adjacent section before microdissection (B), after removing the epithelial top layer (C), after removing the epithelial bottom layer (D), and after removing the stroma (E). Microdissection was always performed in this order. See Supplementary Data for all other cases.

Figure 5.

From left to right, 500 ng RNA each from one FFPE case (0008) and the two epithelial layers from an MFPE case (0043) run on a 0.8% agarose gel. The TrackIt 1Kb Plus DNA Ladder (Life Technologies) is on the right. In the MFPE samples, the 18S band appears distinctly, while the 28S band is less well-defined. The FFPE samples in comparison are completely degraded.

Figure 6.

Plot of expression data from two aliquots of case 0043 basal layer. One aliquot had 200 ng RNA starting material in the labelling reaction (ordinate) while the other used only 50 ng (abscissa). Most data points lie along the linear regression line ($R^2 = 0.96$). Data from saturated spots have been removed (e.g., 200 ng > 2400).

Figure 7.

Plot of expression data from two aliquots of case 0028 basal layer. One aliquot had 200 ng RNA starting material in the labelling reaction (ordinate) while the other used only 25 ng (abscissa). A distinct side branch in the data is visible, suggesting 25 ng may be too little starting material for reproducible data from MFPE tissue. Data from saturated spots have been removed.

Figure 8.

Overlaid M-A plots of duplicate data for the two MFPE samples. Data have been adjusted so that the mean M is zero. The traces are manually fit double exponentials that can be used as thresholds separating data that is indistinguishable from technical variability and likely differentially expressed targets.

Figure 9.

Cluster analysis of all log-transformed microarray data. Pearson distances and complete linkage were used. Each array is labelled by case number (abbreviated as per the legend at right), layer (T = Top, B = Bottom, S = Stroma), grade (Norm = Normal), and the amount of RNA used in the labelling reaction. Where given, the number after the @ symbol denotes the detector gain setting on the microarray scanner. In all other cases (plus the one where gain = 433), the detector gain was set automatically by the scanning software. Two samples that differ only by detector gain were the same hybridized array imaged twice with different settings. All other samples represent distinct arrays. Two 200 ng aliquots of top layer RNA were assayed from case 0044 (case 7 in this figure), so these are denoted A and B. Note that in this figure, cases 4 and 5 are from the same patient, as are cases 8 and 9.

Table 1
Implied false discovery rates attributable to technical scatter

False discovery rates when differential expression criteria based on the scatter of duplicate data are applied to the two sets of duplicate data. For each condition, the counts and percentages of data points outside the indicated threshold bands are given. Refer to text for details on the threshold bands and extra fold-change and intensity criteria.

Threshold bands	Number of false discoveries in duplicate samples	
	Normal	CIN I
Wide	16 (0.0390%)	4 (0.0098%)
Narrow	120 (0.2927%)	43 (0.1049%)
Narrow with extra criteria	63 (0.1537%)	20 (0.0488%)