A New Tissue Stabilisation Technology for High-Quality Tissue-Based Molecular Studies

Medical University of Graz

Viertler C[1], Groelz D[2], Kashofer K[1], Gündisch S[3], Riegman P[4], Winther R[5], Wyrich R[6], Kruhoffer M[7], Becker KF[3], Oelmüller U[6], Zatloukal K[1]

[1]Institute of Pathology, Medical University of Graz, Austria, [2]PreAnalytiX GmbH, Hombrechtikon, Switzerland, [3]Institute of Pathology, Technical University of Munich, Germany, [4]Department of Pathology, Erasmus MC, Rotterdam, The Netherlands, [5]Dako Denmark A/S, Glostrup, Denmark, [6]QIAGEN GmbH, Hilden, Germany, [7]AROS Applied Biotechnology A/S, Aarhus, Denmark

Background

Molecular characterization of human disease relies on the analysis of multiple parameters, from morphological features to a broad spectrum of biomolecules. Histopathological diagnosis is routinely based on formaldehyde-fixed and paraffin-embedded (FFPE) tissues but it is known that formalin fixation impairs molecular analyses which typically require frozen tissue samples. Within the European FP7 project SPIDIA (**www.spidia.eu**) we developed and tested a new technology for combined histological and molecular analyses from paraffin-embedded tissue samples (PAXgene Tissue) and evaluated the impact of several pre-analytical variables on the quality of tissue-based molecular studies.

Methods

Matched samples from different human (non-) malignant tissues were fixed in buffered formaldehyde, non-crosslinking fixatives including the PAXgene Tissue System and paraffin-embedded (PFPE), and snap-frozen tissue samples served as reference. In a comparative study the quality of morphology, antigenicity and different biomolecules was investigated, in particular of nucleic acids.

Results

Preservation of morphology and antigenicity

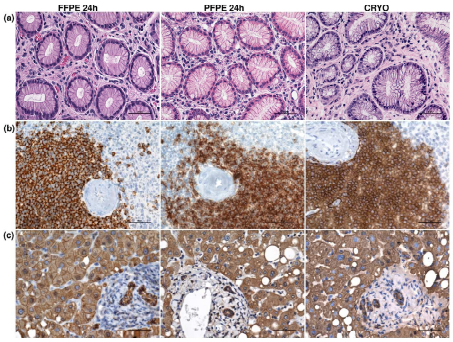


Fig. 1 (a-c) Human tissue samples were fixed in neutral buffered formalin for 24h (FFPE), in PAXgene Tissue Fixative for 24h (followed by 24h of PAXgene Tissue Stabilization; PFPE) or snap-frozen (CRYO).

(a) PFPE samples show well-preserved morphology, comparable to FFPE samples. Standard H&E staining of human stomach tissue.

(b) Comparable preservation of antigenicity in PFPE and FFPE samples. Routinely used diagnostic antibodies were tested on different PFPE tissues, e,g, CD 20 staining of human spleen tissue.

(c) For some antigens less harsh retrieval procedures can be used for PFPE tissues, e.g. for K8/18 staining of human liver .

RNA preservation and gene expression

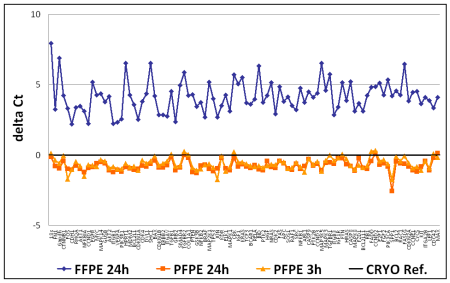


Fig. 2 High correlation of gene signature in PFPE and cryo-preserved samples (R^2=0,99), **whereas FFPE samples show a decreased correlation** (R^2=0,89) **and major gene-to-gene variations.** Gene expression of 96 cancer pathway-associated genes from PFPE, FFPE, and snap-frozen human liver analyzed by qRT-PCR on predefined TaqMan array "Human Molecular Mechanisms of Cancer" plate. Delta Cts were calculated using the frozen sample as reference.

Impact of pre-analytical variables on tissue sample quality and subsequent molecular analyses

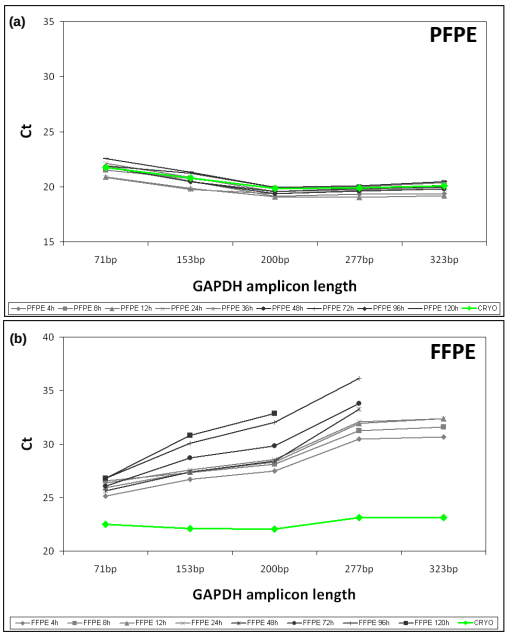
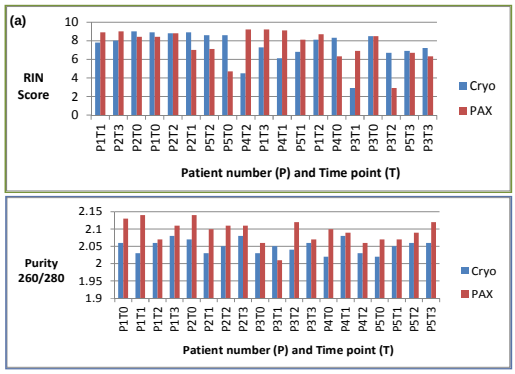


Fig. 3 Impact of type of fixation and time in fixation on RNA quality and PCR efficiency. Human liver samples fixed 4-120h in PAXgene Tissue or formalin. Quantitative real-time PCR assay based on different amplicon lengths of the housekeeping gene GAPDH (71-323bp).

(a) RNA extracted from PFPE samples showed RIN values from 4,8 to 6,1 and comparable qRT-PCR performance with the cryo-preserved reference (green line), independent of time in fixation.

(b) RIN values for all FFPE samples were comparably low (from 2,1 to 2,7), but qRT-PCR analysis revealed that a prolonged time in fixation leads to a rise of ct-values of all amplicons and impairs amplification of longer fragments.



(b)

Fig. 4 Impact of ischemia time and stabilization method analysed with Affymetix Human Genome U219 Array on the GeneTitan hybridization, wash and scanning station. Liver needle biopsies from 5 patients (P1-5), exposed to different ischemia time points (T0-3), were either snap-frozen (Cryo) or fixed and stabilised with PAXgene Tissue (PAX).

(a) Quality control using Agilent Bioanalyzer shows comparable RNA integrity (RIN score) and purity (OD 260/280 ratio) of PFPE and cryo-preserved samples.

(b) GDM analysis showing the Euclidian distance between samples based on expression values from all genes. The variable that contributes most to the distance between samples is the individual and not ischemia time or sample stabilization method.

DNA integrity and performance in multiplex PCR

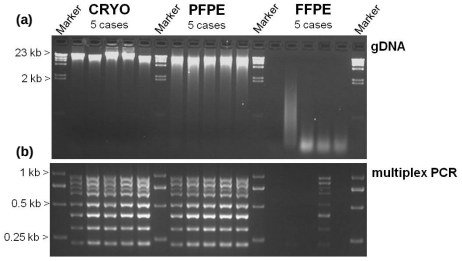


Fig. 5 DNA integrity and performance in multiplex PCR of PFPE samples.

(a) High molecular mass bands indicating good DNA integrity were visible for PFPE and cryo-preserved samples but not for FFPE samples. Genomic DNA extracted from corresponding FFPE, PFPE, and snap-frozen (CRYO) samples of 5 human breast cancer cases was separated on 1% agarose gels and visualized with ethidium bromide.

(b) Multiplex PCR of eight fragments of different human genes. Prion protein (PRNP, 222 bp), immunoglobulin-associated beta protein (CD79b, 310 bp), Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (cKIT, 414 bp), angiotensin II receptor, type 2 (AGTR2, 523 bp), CD14 molecule (CD14, 662), CD40 molecule (CD40, 756 bp), CD59 molecule (CD59, 845 bp), and CD19 molecule gene (CD19, 955 bp).

Western Blot analysis of phosphoprotein preservation

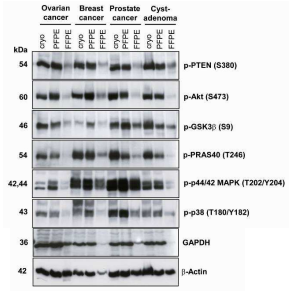


Fig. 6 Preservation of phosphoproteins in PFPE human tissue samples: **visible protein bands of PFPE extracts are comparable to snap frozen samples and the phosphorylation levels are well-preserved.** After protein extraction from corresponding FFPE, PFPE and cryopreserved (non-) malignant human tissue samples, 15 µg protein per lane was separated by SDS-PAGE and western blot analysis was performed using indicated antibodies.

Conclusions

New tissue stabilisation technology PAXgene Tissue provides

- Preservation of **morphology and antigenicity** resembling FFPE samples.
- Excellent **RNA** quality and strong correlation of multiple mRNA profiles with snap frozen samples in qPCR and microarray analysis.
- High molecular mass **DNA**, well-suited for long-range and multiplex PCR, and different sequencing techniques.
- Comparable preservation of **(phospho-) proteins** with snap- frozen samples.
- Improvements of critical **pre-analytical variables** and reliability of tissue-based molecular studies

New opportunities for combined morphological and molecular analyses in different application scenarios

- clinical trials and biomedical research,
- multimodal biomarker studies in a routine clinical setting,
- molecular analyses of lesions where a collection of snap-frozen material is impossible for medical, ethical or logistic reasons.

Contact & further information: christian.viertler@medunigraz.at; Viertler et al. J Mol Diagn. 2012 Sep;14(5):458-66. Epub 2012 Jun 28.