Gene expression profiling of archived FFPE samples

Silvia Rüberg, Sabine Classen, Jana Ciomperlik, Dirk Dietrich, Ines Dischinger, Alena Böttcher, Sabrina Schmitz, Bernhard Gerstmayer
Miltenyi Biotec GmbH, Friedrich-Ebert-Str. 68, 51429 Bergisch Gladbach, Germany

Introduction

According to the BBMRI (Biobanking and Biomolecular Resources Research Infrastructure) about 8,000,000 formalin-fixed paraffin-embedded (FFPE) samples derived from a multitude of different diseases have been collected in medical centers and biobanks all over Europe during the last decades. These samples represent a rich and valuable source that contains disease-relevant genetic and genomic information. However, molecular analysis of FFPE samples has been hampered not only due to difficulties in recovering cross-linked and partially degraded RNA from the respective tissues, but also because of the unavailability of suitable amplification and labeling protocols. In this study, we prepared total RNA from paired samples of FFPE and fresh frozen tissues. In addition, we performed biological and technical replicate experiments and analyzed robustness and reproducibility of different amplification and labeling protocols using samples that have been stored for different time periods.

Methods

RNA was isolated using standard RNA extraction protocols (Absolutely RNA FFPE Kit for FFPE tissues (Stratagene) and Trizol (Sigma) for fresh frozen tissue). The quality and quantity of isolated RNAs was checked on an Agilent 2100 Bioanalyzer and ND-1000 Spectrophotometer (NanoDrop Technologies), respectively. RNA samples derived from FFPE tissues were amplified using the TransPlex Whole Transcriptome Amplification Kit (WTA, Sigma) and subsequently Cy3-labeled using the Genomic DNA ULS Labeling Kit (Agilent Technologies). RNA from fresh frozen tissue was T7-amplified and labeled according to Low RNA Input Linear Amp Kit (Agilent Technologies). 4x44K Agilent Whole Genome Microarrays, One-Color, were scanned with an Agilent Scanner and analyzed using Feature Extraction software (FES). FES-derived data files were used as input for downstream analysis using the RESOLVER (Rosetta Biosoftware) or in-house software AgiRJoiner, Correlate, and Venn (Miltenyi Biotec).

Results

1 Experimental overview and quality control of RNAs

An overview of the experimental set up and workflow is shown in figure 1A. At least two 10-µm slices of three consecutive FFPE sections both from mouse kidney and muscle were used (storage condition: 4 weeks at 4 °C). In addition, fresh frozen kidney and muscle tissues, each in duplicates from the same mouse, were used as gold standard. Furthermore, two different FFPE human melanoma samples were analyzed (storage condition: >5 years at 4 °C). Different amplification and labeling procedures for paired FFPE and fresh frozen samples were hybridized on Agilent microarrays. Finally, data sets were cross-compared among tissues and amplification technologies.
In figure 1B (gel) and C (electropherogram) the results of the Bioanalyzer run are shown. All RNAs derived from murine kidney and muscle FFPE samples revealed RNA integrity (RIN) values <3. The FFPE melanoma samples showed the least integrity with RIN values <2. In contrast, RNA derived from fresh frozen samples showed no signs of degradation and revealed RIN values ~9.

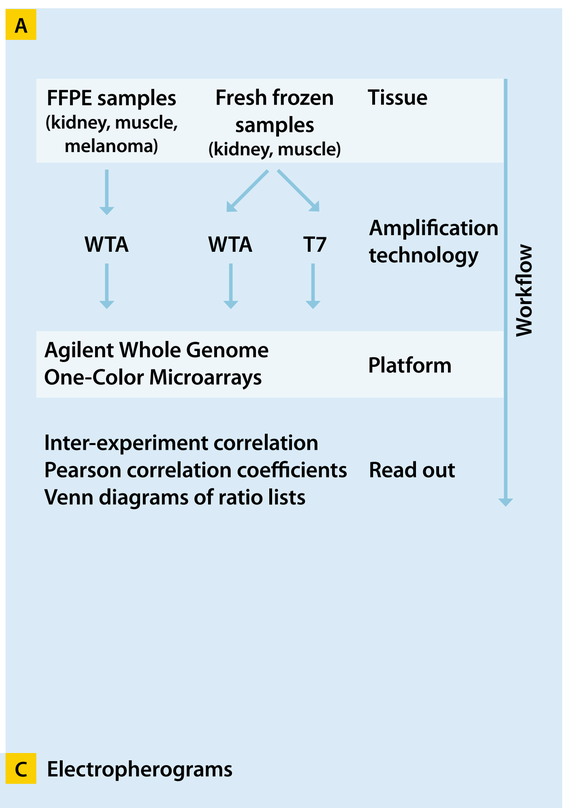
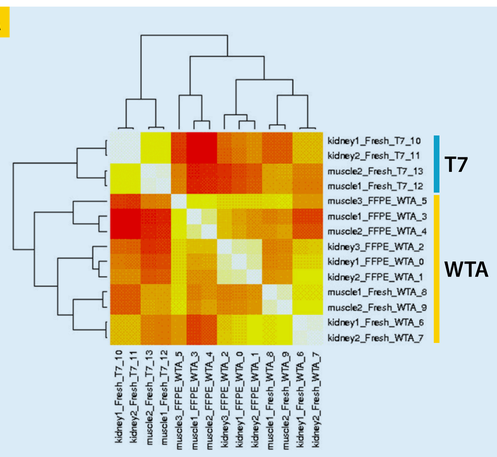


Figure 1

2 Gene expression profiling of FFPE samples is feasible and showed high concordance amongst replicates

Amplified and labeled aRNAs/cDNAs were hybridized, scanned, and feature-extracted. Intensity profiles derived from mouse FFPE tissues were subjected to a correlation analysis using Euclidian distances. Figure 2A shows the correlation values. Although intensity profiles were separated into different clusters depending on the amplification type (WTA or T7), a high correlation of technical replicates could be observed (fig. 2B; bold numbers indicate the correlation coefficients of technical replicates). To check for signal sensitivity of the respective amplified samples in the data sets, we analyzed the number of "present calls" for fresh and FFPE kidney samples as well as for >5-year-old FFPE melanoma samples (determined by "gIsPosAndSignif" values from FES). Figure 2C indicates that the amplification technology had no effect on sensitivity. Furthermore, there was no significant difference in the number of present calls from differently stored FFPE samples. As anticipated, the number of detectable genes from FFPE tissue versus fresh frozen tissues was lower (approx. 20%).



C

Tissue type	Present calls (%)	Storage conditions
Kidney1_FFPE_WTA	62.2	FFPE (4 weeks)
Kidney2_FFPE_WTA	63.9	FFPE (4 weeks)
Melanoma1_FFPE_WTA	64.8	FFPE (> 5 years)
Melanoma2_FFPE_WTA	57.1	FFPE (> 5 years)
Kidney1_Fresh_WTA	79.6	Fresh frozen
Kidney2_Fresh_WTA	80.3	Fresh frozen
Kidney1_Fresh_T7	80.5	Fresh frozen
Kidney2_Fresh_T7	81.3	Fresh frozen

B

	k1_ FFPE_ WTA	k2_ FFPE_ WTA	k3_ FFPE_ WTA	m1_ FFPE_ WTA	m2_ FFPE_ WTA	m3_ FFPE_ WTA	k1_ Fresh_ WTA	k2_ Fresh_ WTA	m1_ Fresh_ WTA	m2_ Fresh_ WTA	k1_ Fresh_ T7	k2_ Fresh_ T7	m1_ Fresh_ T7	m2_ Fresh_ T7
kidney1_FFPE_WTA	1.00													
kidney2_FFPE_WTA	**0.96**	1.00												
kidney3_FFPE_WTA	**0.96**	**0.95**	1.00											
muscle1_FFPE_WTA	0.74	0.71	0.78	1.00										
muscle2_FFPE_WTA	0.74	0.71	0.78	**0.94**	1.00									
muscle3_FFPE_WTA	0.82	0.82	0.82	**0.88**	**0.89**	1.00								
kidney1_Fresh_WTA	0.82	0.85	0.80	0.57	0.60	0.72	1.00							
kidney2_Fresh_WTA	0.82	0.85	0.80	0.57	0.60	0.72	**0.99**	1.00						
muscle1_Fresh_WTA	0.69	0.71	0.68	0.68	0.71	0.82	0.84	0.84	1.00					
muscle2_Fresh_WTA	0.70	0.72	0.69	0.70	0.73	0.84	0.85	0.85	**0.97**	1.00				
kidney1_Fresh_T7	0.67	0.70	0.63	0.41	0.42	0.56	0.77	0.77	0.61	0.62	1.00			
kidney2_Fresh_T7	0.67	0.70	0.63	0.41	0.42	0.56	0.78	0.78	0.62	0.62	**0.99**	1.00		
muscle1_Fresh_T7	0.59	0.61	0.55	0.53	0.54	0.68	0.68	0.68	0.74	0.74	0.87	0.87	1.00	
muscle2_Fresh_T7	0.58	0.61	0.55	0.52	0.52	0.67	0.68	0.68	0.74	0.74	0.87	0.87	**0.99**	1.00

Figure 2

3 FFPE samples and fresh frozen samples reveal similar gene regulation

To investigate whether there is a correlation between the genes that are found to be regulated in different FFPE tissues and fresh frozen samples, ratios from intensity profiles of "kidney-versus-muscle" were calculated. In addition, we investigated the overlap between differentially expressed genes derived from the different amplification technologies using the same starting material. Only genes with a >2-fold change and a p value <0.01 were used for VENN analysis. As outlined in figure 3, the majority of differentially regulated genes found in FFPE "kidney-versus-muscle" tissues could also be found in fresh frozen tissue (pearson correlation coefficient: 0.67). Furthermore, a concordance of ~70% of differentially expressed genes between the same samples that have been amplified with T7 or WTA could be observed (pearson correlation coefficient: 0.78).

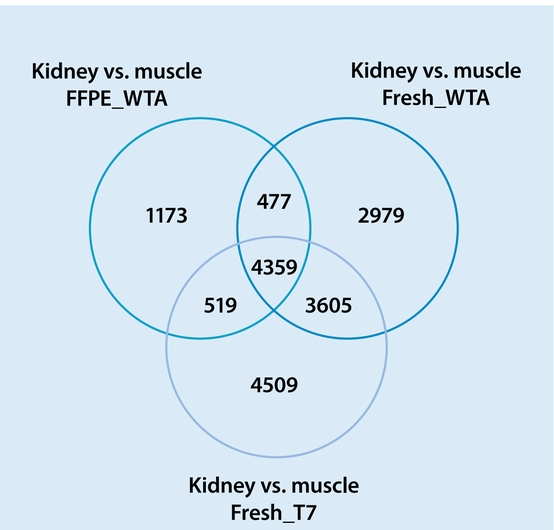


Figure 3

Conclusion

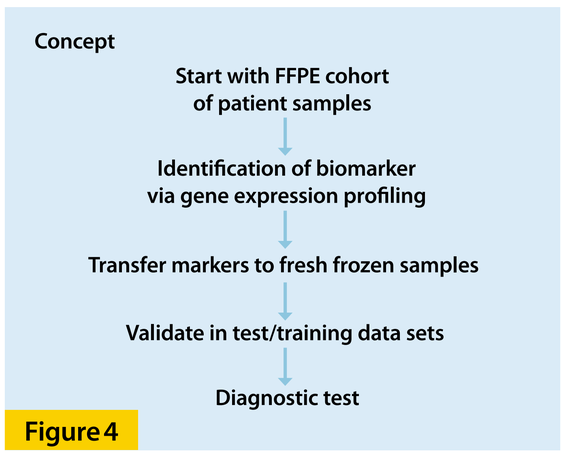


Figure 4

- The novel FFPE protocol for RNA isolation and amplification is feasible and revealed highly reproducible results amongst replicates.
  Even very old FFPE samples could be successfully analyzed.
- As expected, the signal intensities and the number of detectable genes is somewhat lower in FFPE compared to fresh frozen samples, which is most likely due to a failure of recovery and amplification of a certain percentage of transcripts from FFPE tissues.
- The amplification technology and type of fixation/preservation of tissues in the current experimental setting overrules the biological differences of the two tissue types. Therefore, our recommendation is to keep the amplification or fixation type constant during a gene expression profiling project.
- Nevertheless, due to a high overlap of differentially expressed genes found in FFPE and corresponding fresh frozen samples, gene expression profiling of FFPE samples represents a new promising approach. The easy access to FFPE tissues and the availability of clinical outcome data makes an integrative analysis for prognostic/diagnostic biomarker prediction very attractive (fig. 4).

Reference
Hoshida, Y. *et al.* (2008) N. Engl. J. Med. 359: 1995–2004.

For further information on our FFPE Microarray Services please contact
macstec@miltenyibiotec.de