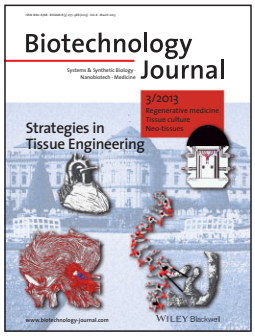

Cover illustration

Tissue engineering is an integrative field that combines knowledge from several disciplines such as cell biology, biochemistry, medicine, and material sciences. The cover of this Special Issue on *Strategies in Tissue Engineering* – showing, an organ, a tissue sample, a bioreactor and a DNA strand, represents the holistic nature of this discipline and the multitude of expertise necessary to drive it forward. Forming the background to the image is the Würzburg Residence, a UNESCO World Heritage Site in Bavaria , Germany , and the location of the 3rd International Conferences "Strategies in Tissue Engineering". © Katja Schenke-Layland.

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Technical Report

RNA isolation from fetal and adult human tissues for transcriptional profiling

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Investigations involving rare human tissues that are difficult to acquire due to their scarcity are highly challenging. The need to verify microarray analysis data by additional methods such as immunohistochemical staining and quantitative PCR creates an even greater demand for these valuable tissues. Furthermore, since rare human tissues may come from different sources and may have been processed by variable methods, the comparability of these samples must be verified. The aim of this study was to determine and validate a processing method that allows the analysis of human fetal and adult cardiovascular tissues from different sources that were preserved using varying methods. Due to restricted access to fresh human tissues and the need to accumulate these samples over an extended period of time, we used formalin-fixed paraffin-embedded tissues for gene expression analyses. We analyzed RNA levels from four different age groups: fetal first and second trimester, adolescents, and adults. In this study, we present an improved standard processing procedure for tissue sample processing and analysis of rare human cardiovascular tissues.

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1 Introduction

The past decade has seen a greater focus on rare disease research and investigations involving human fetal tissues [1–3]. Furthermore, as the world's population ages, medical sciences are being pressured to translate animal-model based discoveries into clinical applications, which will subsequently inspire more work with rare human tissues. With the European Union directive of Replace, Refine, and Reduce coming into enforcement [4], the reduction of allowable animal studies and the limited availability of human tissues will further exacerbate the problem of tissue access. Therefore, it will be essential to optimize and verify the preservation and processing methods used to analyze human tissues.

The two standard methods to preserve tissues and biopsies are formalin-fixation and paraffin embedding (FFPE) and cryopreservation. Most of the biopsies currently stored in laboratories and hospitals are FFPE blocks. Routinely, FFPE samples are used for morphological, histolog-

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Abbreviations: DASL, cDNA-mediated annealing, selection, extension, and ligation; **DPBS**, Dulbecco's phosphate-buffered saline; **FFPE**, formalin-fixed paraffin-embedded; **LCM**, laser-capture microdissection; **PCA**, principle component analysis; **RIN**, RNA integrity number

ical, and immunohistological analyses. Researchers all over the world employ this relatively simple standard method to preserve the tissue architecture as well as the cell morphology and biochemical components for long-term storage. Thus, the archived samples provide a unique source to study time and location dependent cellular and extracellular processes in order to reveal pathological mechanism and identify new biomarkers [5]. For clinical application, human donor heart valves are routinely cryopreserved to be used as homografts. The cryopreserved valves are stored in homograft banks prior transplantation. Fresh harvested valves are first sterilized in antibiotics-containing solutions for 1 day, which are then placed into cryoprotectants to be preserved by freezing. Thus, slow-rate freezing protocolls are established to freeze the valves with a controlled-rate until –196°C (vapor phase of liquid nitrogen) is attained [6, 7].

In this study, we analyzed FFPE preserved human fetal first and second trimester semilunar heart valves, isolated via laser-capture microdissection (LCM), and cryopreserved/FFPE preserved human adolescent and adult semilunar heart valves. We analyzed the quantity and quality of the acquired RNA and employed the Illumina cDNA-mediated annealing, selection, extension, and ligation (DASL)-assay, which is a microarray platform for partially degraded RNA, for global gene analysis. We performed statistical analyses to ensure the comparability of tissues from vastly different age groups and modified processing methods.

2 Materials and methods

2.1 Tissue procurement and processing

This study was performed in accordance with institutional guidelines and was approved by the local research ethics committees (UCLA IRB #05-10-093; University Tübingen IRB #356/2008BO2). First and second trimester (8–17 wk of gestation) human fetal hearts (n = 15) were obtained from discarded material obtained from elective terminations of pregnancies performed by Family Planning Associates in Los Angeles. Cryopreserved aortic valves (n = 8), which were not suitable for transplantation due to extended storage times, were provided by Cell and Tissue Systems (Charleston, USA). After either harvest or thawing, all tissues were washed in sterile Dulbecco's phosphate buffered saline (DPBS) prior further processing.

2.2 FFPE and cryopreservation

The tissues were fixed in 10% phosphate-buffered formalin after no longer than 6 h in DPBS at 4°C. Importantly, the fixation time was for a maximum of 12 h at room temperature. Afterwards, all tissues were rinsed in distilled water and transferred to 70% ethanol. Fixed tissues were embedded in paraffin and stored at room temperature for up to 3 years. Heart valves were processed according to a conventional cryopreservation protocol by freezing disclosed in U.S. Patent 4890457 [8].

2.3 Preparation of FFPE-tissue sections for LCM

Micrometer sections were cut using a rotational microtome (RM 2145, Leica/Germany). Paraffin sections (thickness: 12 μm) were mounted on RNase/DNase free PEN membrane slides (Carl Zeiss MicroImaging/Germany). In order to ensure an improved attachment to the membrane, mounted slides were incubated at 56°C overnight. A cresyl violet staining was required to visualize the fetal heart valve leaflets. The protocol was modified from the manufacturer's protocol for RNA handling. After paraffin removal, tissues were hydrated in 100, 96, and 70% ethanol for 1 min; each slide was dipped into RNase-free water and stained for 30 s in cresyl violet solution. The slides were then dipped in 70 and 100% ethanol. For LCM, we used a Zeiss P.A.L.M. Micro-Laser System and the corresponding P.A.L.M RoboSoftware (Carl Zeiss).

2.4 Extraction of total RNA from FFPE tissue

Extraction of the total RNA of FFPE-treated heart valve tissue was performed using the FFPE RNeasy Kit from Qiagen (#73504, Qiagen/Germany). The tubes containing the tissue pieces obtained by LCM were incubated in Proteinase K for 16 h at 56°C. The RNA was extracted according to the FFPE RNeasy protocol.

2.5 Extraction of total RNA from cryopreserved tissue

The Microarray RNeasy Kit from Qiagen (#76163, Qiagen/Germany) was used for cryopreserved heart valve tissues, which were not additionally FFPE-treated. The heart valve leaflet was dissected and a maximum of 100 mg was homogenized in QIAzol using a tissue disruptor. The following procedure was performed according to the manufacturer's protocol.

2.6 RNA quantification and qualification

The total RNA was quantified employing the UV spectrometer NanoDrop 2000 (PEQLAB Biotechnologie GmbH/Germany). The quality of the RNA was monitored using automated electrophoreses systems, specifically the 2100 Bioanalyzer from Agilent Technologies. RNA quality was measured by the fluorescence signal, which is associated to the length of the RNA fragments. In general, good quality RNA results in two peaks at the 18S and 28S bands, identifying the small and large ribosomal subunits. Based on the 18S and 28S peaks, the RNA integrity

number (RIN) was calculated. When the RNA integrity is degraded, the 18S and 28S peaks diminish. The length of the remaining RNA fragments can then be used to determine the RNA quality [9].

2.7 Microarray

Whole genome expression analysis was performed by MFT services (University of Tübingen) using a Whole-Genome DASL-Array (Illumina, Inc., San Diego, USA), which is specifically designed for RNA of FFPE processed tissues. One hundred nanograms of total RNA was hybridized to the array. Priming was performed with random hexamers as these give higher detection rates from FFPE than oligo(dT) primers [10]. After cDNA synthesis and biotinylation, the cDNA was connected with assay-specific oligonucleotides to bind to paramagnetic beads. After PCR using fluorescence-labeled primers, the amplification products were hybridized to the Whole-Genome BeadChip and scanned by the BeadArray Reader (Illumina Inc., San Diego, USA).

2.8 Statistical analysis

Averaging of intensities of replicate beads and quality control was performed with Genome Studio V2009.1 software (Illumina, San Diego, CA). No background correction or normalization was performed at this stage [11]. Summarized intensities together with standard errors, number of beads per bead type, and detection *p*-values were exported. All subsequent data analysis steps were performed on the software platform R 2.12.0 and Bioconductor 2.6.1 [12] with the packages "beadarray" [13, 14], "limma" [15], "RankProd" [16], and "GOstats" [17] as well as Partek 6.5. Initially, the expression data from all chips were normalized with variance stabilizing normalization (VSN) [10]. For principle component analysis (PCA) and boxplot analysis we used Partek 6.5 software.

Statistical significance was determined by ANOVA Tukey's multiple comparison tests and *t*-test using GraphPad Prism 5 software (GraphPad Software Inc, San Diego, CA, USA). *p*-values <0.05 were defined as statistically significant.

3 Results

Prior to sectioning, we cleaned the microtome and forceps with RNase-Zap and used RNase-free water for the stretch-bath. The slides were incubated at 56°C overnight to obtain better attachment of the paraffin sections to the RNase-free membrane slides. We identified that these slides must be placed in a horizontal position to avoid tissue disruption caused by the fluid paraffin. Ideally, the quick routine stain cresyl violet was sufficient to visualize the heart valve leaflets. The leaflets could be easily distinguished from the vessel wall and myocardium (Fig. 1).

A 5× objective was employed to observe second trimester leaflets, whereas first trimester fetal leaflets required 10× or 20× objectives in order to be able to cut out the region of interest accurately. We determined a thickness of 12 μm of paraffin sections as optimal for our

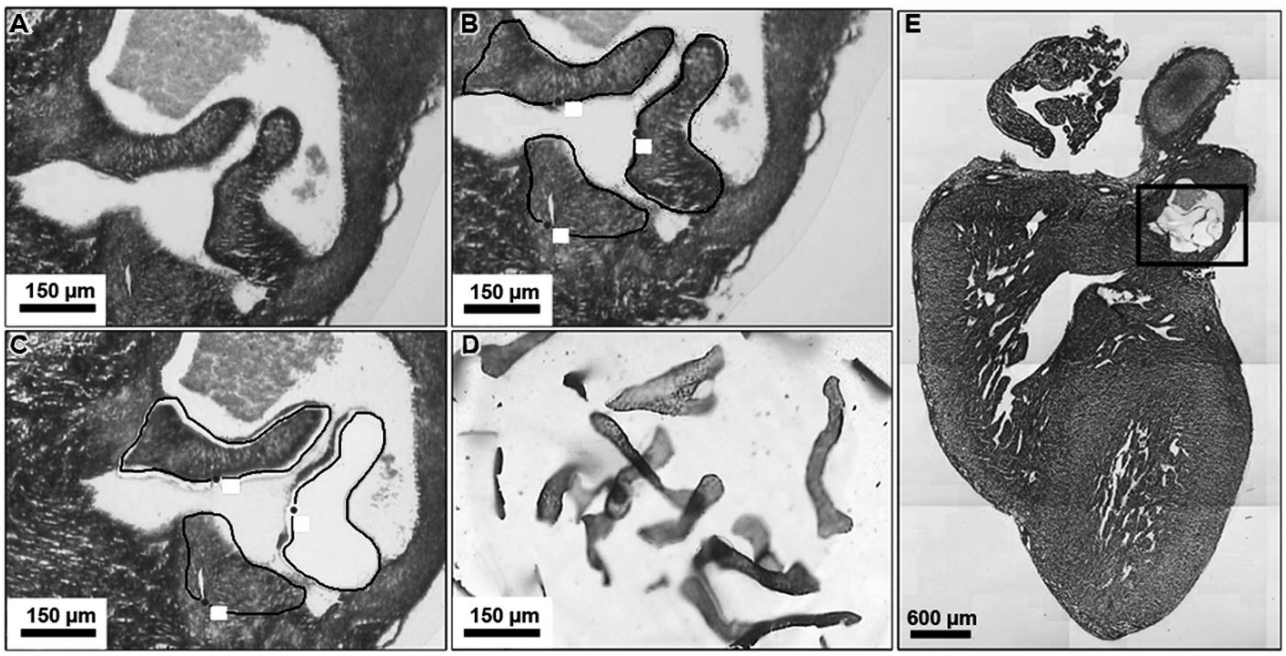

Figure 1. Cresyl violet staining of fetal hearts allows the identification of the valve leaflets, which are marked, cut and catapulted by LCM. (**A**) Heart valve leaflets, (**B**) marked area, (**C**) after cutting one leaflet, (**D**) collected leaflets in the cap, and (**E**) fetal heart section after LCM of valve leaflets.

cutting area. This was a compromise between optimal tissue yield and thickness of the sections to be catapulted without failure. The 5× objective particularly required that the catapulted areas were limited in size, as too large areas could not be lifted into the capturing cap. For this reason, mature heart valve leaflets were directly cut with a scalpel and transferred to an RNase-free tube.

The RNA integrity was assessed using the Bioanalyzer. As reflected in the spectra, compared to RNA obtained from fresh tissues (Fig. 2A), the integrity of FFPE RNA was significantly decreased (Fig. 2B). RNA of vital tissues contained a 18S and 28S band and exhibited RINs of 9–10 [18]. Tissue processing reduced the RNA quality due to degradation and cross-linking so that only RNA fragments remained. In this study, all FFPE samples exhibited RINs in a range of 1.9–2.5 (mean 2.2 ± 0.19). In addition, we extracted RNA from cryopreserved leaflets directly after the thawing procedure (Fig. 2C). The RNA quality of the cryopreserved samples was significantly lower compared to normal fresh or snap-frozen tissues. We found that cryopreserved tissues exhibited only low integrity numbers (mean RIN 3.9 ± 0.8); however, the fluorescence detection time was increased compared to FFPE RNA, and interestingly, both 18S and 28S bands were detectable in these tissues.

In order to be able to directly compare RNA qualities, we formalin-fixed and embedded the cryopreserved leaflets in paraffin. We identified that the RNA quality of these tissues was comparable to the RNA quality of the fetal FFPE samples, ranging between RINs of 1.9 and 2.5.

In order to ensure sufficient amounts of RNA, the required amount of tissue had to be adapted. In Fig. 2D, the RNA yield is depicted. Accordingly, we were able to extract approximately 0.0041 pg RNA from each 1 μm^3 of a FFPE heart valve leaflet from fetal hearts within the first trimester of gestation. Interestingly, the obtainable RNA amount decreased significantly in the second trimester tissues (0.0011 pg/μm^3; $p < 0.0001$). Additionally, in adolescent leaflets the mean RNA amount decreased to 0.00031 pg/μm^3, and to 0.00021 pg/μm^3 in adult tissues.

We compared global genome signal intensities of all samples. The non-normalized data revealed that the overall signal intensity was increased in all cryopreserved mature leaflets (Fig. 3A). As expected, additional processing steps resulted in lower overall signal intensities, which can be explained by the presence of lower amounts of transcripts in these samples. Using normalization, the signal distribution within the different samples (cryopreserved and FFPE) was similar, which means that the samples are coherent among each other. In detail, all samples exhibited similar amounts of highly expressed and lower expressed genes (Fig. 3B). The median of all samples was within a similar range, with exception of the FFPE-treated cryopreserved mature leaflets. Four of eight samples exhibited a decreased median, which implies that these samples included a higher amount of lower signal intensities, and only fewer transcripts could be detected. For this reason, these samples had to be specifically monitored and were compared with the corresponding cryopreserved tissues.

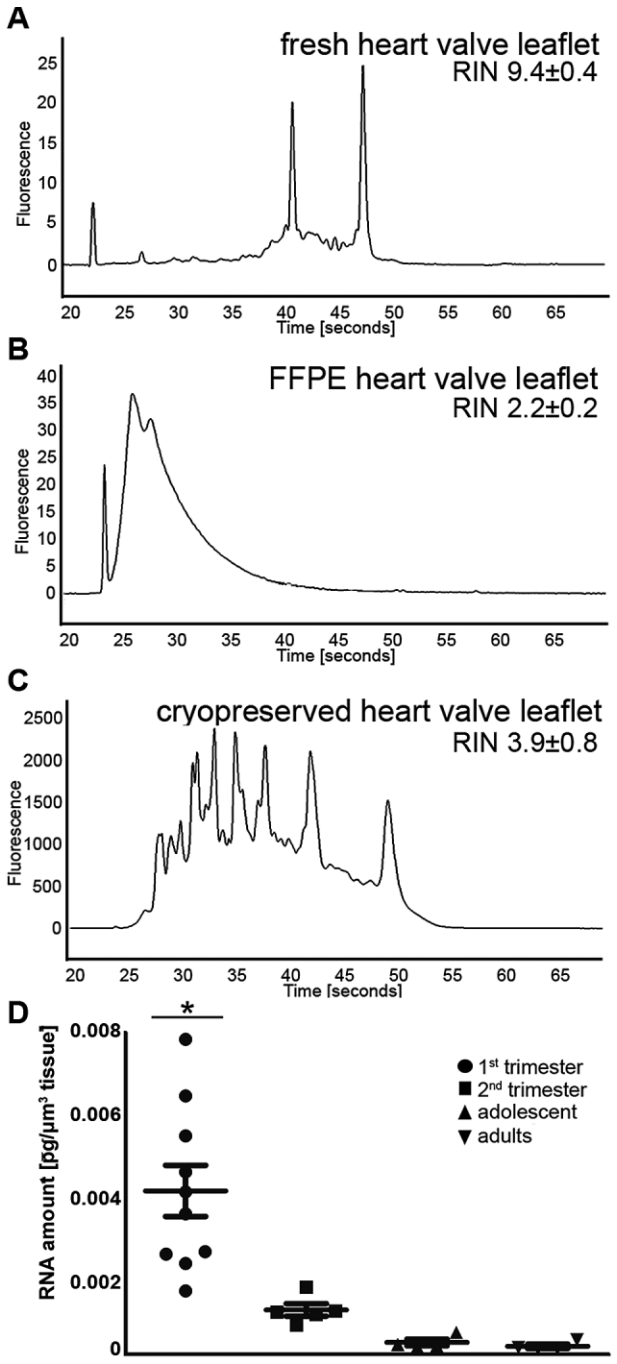

Figure 2. Bioanalyzer data of RNA extracted from (**A**) fresh ($n = 4$), (**B**) FFPE ($n = 15$), and (**C**) cryopreserved ($n = 8$) heart valve leaflets. (**D**) Extracted RNA amount from 1 μm^3 FFPE tissue of the different age groups: fetal first ($n = 10$) and second trimester ($n = 5$) as well as adolescent ($n = 4$) and adult ($n = 4$). *$p < 0.0001$ RNA amount [pg/μm^3] of first trimester tissues versus to second trimester, adolescent and adult.

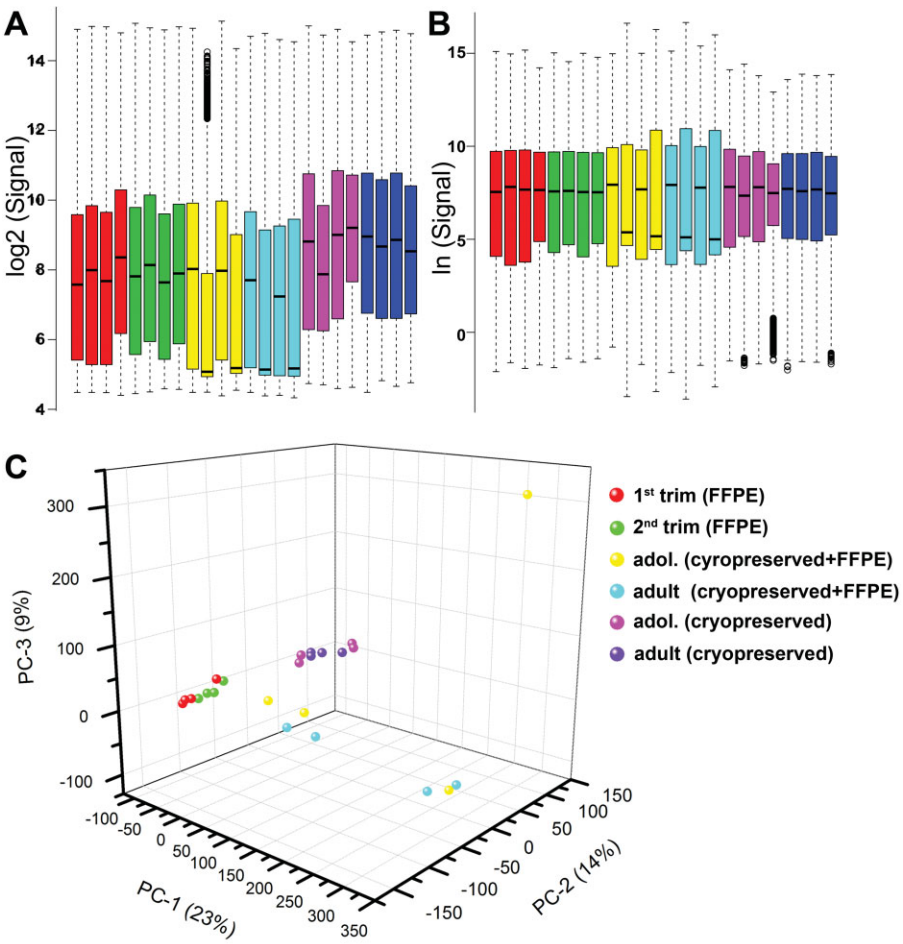

Figure 3. (**A**) Boxplot of non-normalized gene expression data. (**B**) Boxplot of normalized gene expression data. Medians are depicted as a black line within the boxes (**C**). PCA of all samples revealed homogeneity within fetal FFPE tissues and within the cryopreserved adolescent and adult samples, but not within the FFPE-treated cryopreserved tissues (each sample group $n = 4$).

PCA revealed that fetal FFPE samples were homogenous and that cryopreserved samples clustered within a group (Fig. 3C). In contrast, FFPE-treated cryopreserved tissues were more broadly distributed. According to the boxplot analysis, we identified four samples that were more similar to the fetal FFPE samples, whereas three samples appeared to be more separated, and one sample was identified as a complete outlier. Interestingly, the fetal tissues showed the most similarities within their group.

Although the gene expression patterns of cryopreserved and FFPE valves were similar, the genes in cryopreserved valves exhibited generally higher intensities compared to the expression in FFPE tissues. Within non-normalized data, 50.5% of all genes (24 528) in the array were differentially expressed (fold change >2) in the cryopreserved and FFPE samples (data not shown). Approximately 96.2% of these genes were decreased in FFPE tissues. In contrast, only 1.9% of all genes showed a higher expression in FFPE-treated cryopreserved tissues when compared to the cryopreserved tissues. Normalization was used to match the signal intensities of the different treatments. Accordingly, only 1372 genes (5.6%) were expressed differently (fold change >2). Most of these genes (4% of all genes (= 989 genes)) were expressed higher in cryopreserved compared to FFPE-treated tissues.

4 Discussion

To date, most developmental studies are performed in small animal models, such as mice, chicken, and zebrafish. Although these studies are highly relevant for our basic understanding and knowledge of molecular mechanisms and interactions, not all data can be directly translated to the human system. In this study, we aimed to develop a strategy to perform developmental studies using available human tissues.

In order to obtain global gene expression patterns of different time points within human development (fetal and mature), we performed microarray analyses. For comparative global gene expression analyses one crucial factor is to provide RNA of similar quality [19]. Indeed, RNA of good quality (RIN 8–10) facilitates routine gene expression studies, enabling more application opportunities compared to RNA of poor quality [18]. Microarray platforms such as the DASL array are particularly designed for

partially degraded RNA to overcome issues associated with poor RNA quality. The availability of fresh fetal and mature human tissues is highly restricted. Currently, we have access to pre-treated tissues, which means cryopreserved or FFPE-treated tissues. Although the RNA integrity of these samples is modest, it is the only option to perform transcriptional profiling analyses of developing and mature human heart valve tissues.

In FFPE RNA, usually no 18S and 28S bands are detectable and the RINs are typically around 2, which reflects fragmentation and chemical modification due to the pre-treatments [9, 20, 21]. The RNA we had obtained in this study from FFPE heart valve leaflets from both fresh fetal and cryopreserved mature tissues, exhibited RINs ranging between 1.9 and 2.5. Previous studies demonstrated that the warm ischemia and formalin-fixation time are the most critical steps for maintaining the RNA integrity [22]. As formalin is known to penetrate 0.5 mm/h [23], we reduced the fixation time depending on the tissue size to a maximum of 12 h. Formalin-fixation cross-links the RNA molecules, which consequently break into fragments. Therefore, this is rather a fragmentation than a degradation process, which implicates that the transcriptional information of the mRNA still remains in the smaller RNA fragments. In order to avoid any RNA degradation due to exogenous RNase, we adapted routine protocols using RNase-free water for sectioning of paraffin blocks as well as RNase-free solutions for deparaffinization and cresyl violet staining. Due to the size of fetal heart valve leaflets, we used LCM to isolate a pure population of leaflet cells. Mature leaflets could be dissected without a microscope using forceps, as it had been previously shown that neither LCM nor scratching affects the RNA integrity [9]. After the collection of FFPE tissue, either through LCM or scratching, all samples were treated the same. Importantly, whereas 18S and 28S bands served as quality criteria for RNA of fresh and frozen tissues, the length of the detected fluorescence was relevant for the evaluation of the FFPE RNA [9]. In order to obtain the required RNA quantity for one DASL array (100 ng per sample), we identified that significantly lower tissue volumes were needed from fetal first trimester leaflets (0.02 mm^3; $p < 0.0001$) when compared to second trimester (0.09 mm^3), adolescent (0.32 mm^3) and adult (0.47 mm^3) leaflets. Due to the rapid growth that occurs in the first trimester development, changes in cell density between early and late first trimester samples resulted in a highly heterogeneous group.

Although routine cryopreservation by freezing is the current gold standard for preserving human tissues for clinical purpose, it is not the optimal procedure for preserving RNA as the explanted valves are not directly frozen, like snap-frozen tissues, which usually serves as RNA control. In contrast, homografts are incubated in antibiotics containing media at 4°C overnight prior to slow, 1°C/min, controlled-rate cryopreservation to –80°C before transfer to the vapor phase of nitrogen. In addition, conventional cryopreservation supports cell viability. Therefore, cryopreserved tissues possess a higher metabolic activity when compared to snap-frozen tissues, which may contribute to an impaired RNA quality. However, compared to the FFPE RNA, the RNA extracted from cryopreserved valves exhibited a lower degradation level. As lower degradation levels of RNA result in a higher detection of gene expression within DASL assays, the RNA without FFPE treatment is expected to result in higher expression intensities [19]. This offers the possibility to monitor which gene expression signals were lost most likely due to the FFPE procedure. Based on the boxplot diagram, our gene expression data showed that the signal intensities of the FFPE samples were generally lower than the non-treated control RNA. In addition, PCA identified that the different samples clustered within their age groups, except for the FFPE-treated cryopreserved adolescent and adult valves. In 50% of these samples, RNA was damaged to a higher degree. Due to the additional processing, lower amounts of the transcripts were detected, which resulted in an overall signal intensity decrease, identified through a decreased boxplot median. Based on this data, we concluded that the FFPE procedure had harmed the RNA more than the other samples. PCA identified one outlier sample within the cryopreserved/FFPE-treated tissues. The reason for the significant difference in gene expression is unclear as we did not detect outliers in the cryopreserved analogs, which means that the tissue sample itself was not affected. However, our experimental set-up and data analyzes were able to detect the outliers. We could determine that one out of two additionally processed samples generated reliable global gene expression data. The comparison of FFPE RNA and RNA from cryopreserved tissues confirmed that different detection of genes is most likely based on additional processing steps.

Certainly, fresh tissues that are not impacted by fixation or cryopreservation would be the first choice to perform transcriptional profiling; however, these tissues are scarce. Thus, FFPE and cryopreserved tissues are the source of human tissues available for research. Although the processing of the tissues translates to challenging RNA integrities, we were able demonstrate that using the appropriate microarray platform, gene expression profiles can be generated, which are suitable for comparative overviews between different developmental stages. However, all RNA-based expression data have to be confirmed and functionally verified on a protein level.

In this study, we established a method for optimal tissue processing in order to obtain FFPE RNA from fetal heart valve leaflets that was suitable for global gene expression profiling. These methodological improvements will allow the recruitment of new tissue sources, in particular with respect to fetal tissues, which will help advancing our current knowledge of human development.

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The authors declare no conflict of interest.

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