Recovery of genomic and transcriptomic profiles from decades-old FFPE brain tissues

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Abstract

Neurological, neurodegenerative, and psychiatric disorders impose substantial morbidity and disability worldwide, yet their molecular basis remains incompletely understood, in part due to limited access to human brain tissue. The Danish Brain Collection, comprising brains from individuals who lived in Danish psychiatric institutions from the 1940s to the 1980s, represents a unique but largely untapped resource for retrospective molecular investigation. Here, we assess the feasibility of extracting and sequencing DNA and RNA from decades-old FFPE brain tissue. We systematically evaluate how extraction and library preparation strategies influence nucleic acid yield and quality, and show that RNA end-repair prior to library preparation substantially enhances transcript diversity, improving data quality from highly degraded samples. Despite extensive fragmentation, we recover biologically informative transcriptomic profiles, including protein-coding and microRNA expression profiles that retain clear tissue specificity. These results establish the Danish Brain Collection as a viable resource for genomic and transcriptomic analyses and demonstrate the broader potential of archival FFPE tissues for large-scale molecular studies.

Keywords: FFPE, brain, RNA, DNA, Danish Brain Collection, End repair.

Introduction

Neurological, neurodegenerative, and psychiatric disorders affect over three billion individuals worldwide, representing one of the most significant public health challenges of our time [1]. These conditions diminish the quality of life of affected individuals and impose enormous economic burdens on families and healthcare systems [2]. Yet our ability to diagnose, predict disease progression, and effectively treat these disorders remains limited. Next-generation sequencing of brain-derived DNA and RNA has begun illuminating the molecular mechanisms underlying conditions like schizophrenia and major depressive disorder [3], Alzheimer's disease

and related dementias [4], or epilepsy [5], revealing disease-associated genetic variants, disrupted pathways, and potential therapeutic targets. Despite these advances, routine clinical protocols still predominantly rely on symptom-based assessment, which carries inherent limitations: symptoms overlap between disorders, early disease presents subtly, and subjective interpretation can lead to diagnostic uncertainty [6]. This introduces the risk of misdiagnosis leading to inappropriate treatment [7]. Moreover, most pathologies remain challenging to treat [8]. Therefore, the current gap between clinical intervention and patients' needs stems from an incomplete molecular understanding of the pathological changes in the brain underlying these diseases.

A major issue faced by researchers is that large-scale molecular studies ideally require fresh tissue in which nucleic acids remain intact. Acquiring such samples is exceptionally difficult due to ethical considerations, logistical challenges, and donor scarcity [9], which hinders our ability to capture the full spectrum of disease heterogeneity. Formalin-fixed paraffin-embedded (FFPE) tissue archives represent a potential alternative. Over the decades, health institutions have accumulated vast FFPE repositories encompassing diverse patient populations, disease states, and historical periods.

One such resource is the Danish Brain Collection (DBC) (https://brain-collection.rsyd.dk/). Assembled between 1945 and 1982, this repository comprises 9,479 brains from Danish psychiatric patients, including an estimated 50,000 FFPE tissue blocks representing multiple brain regions, and spanning widely different treatment eras dominated by divergent infectious disease landscapes and environmental exposures. It ranks among the world's largest brain collections and encompasses an exceptionally broad diagnostic spectrum: dementia, major depression, schizophrenia, epilepsy, Huntington's and Parkinson's disease, among others. Examining molecular signatures across this nearly four-decade window offers unique opportunities to investigate whether brain disorder biology has remained constant or evolved through time, something that contemporary biobanked archives alone cannot easily reproduce. Despite its potential, the Danish Brain Collection remains largely unexplored using modern molecular techniques. No systematic efforts have applied high-throughput sequencing to these samples, likely due to ethics restrictions and concerns about their age and degradation status. Indeed,

formaldehyde fixation is known to create extensive cross-links and fragmentation in biomolecules present in the tissues [10], severely compromising genetic material integrity. For sequencing, these issues translate into low yield, poor library complexity, and high error rates [11]. Even after optimization efforts [12–15], the molecular viability of decades-old FFPE brain tissues archived in pathology collections has remained largely uncharacterized, particularly where fixation protocols were not standardized.

Nevertheless, despite the extensive induced cross-linking and chemical damage created in tissues upon FFPE treatment [16], valuable molecular information can still be recovered [17–20], even from spatially localized areas of a given FFPE block [21–23]. Indeed, compelling evidence suggests that the main limiting factor affecting nucleic acid integrity is not the age of the FFPE sample per se, but rather the chemical conditions with which the original tissues were treated [16,24–28]. Moreover, recent methodological developments have enabled the recovery of genomic and transcriptomic data from specimens up to thousands of years old using specialized protocols handling extreme fragmentation and chemical modifications [29–34]. Therefore, the main question is not whether genetic material may survive over time, but whether molecular information remains sufficiently intact to yield robust biological insights.

In this pilot study, we aimed to determine whether informative high-throughput DNA and RNA sequencing data from a subset of decades-old FFPE brain samples archived in the DBC can be isolated, sequenced and analyzed, so as to reconstruct tissue-specific gene expression patterns of the original human brain tissues. To do so, we first worked on optimizing laboratory methods for DNA and RNA extraction and library preparation and then applied custom bioinformatic pipelines inspired by previous reports on historical archived tissues from museum collections [32].

Methods

Study design

We used human post-mortem brain tissue in the form of formalin-fixed paraffin-embedded (FFPE) tissue archived at The Danish Brain Collection, which is located at Brain Research - Inter Disciplinary Guided Excellence (BRIDGE), University of Southern Denmark, OUH, the Region Psychiatry of Southern Denmark, Odense, Denmark. The Danish Brain Collection is a biobank comprising 9,479 brains, which were obtained during autopsies conducted at Danish state mental hospitals from 1945 to 1982 (https://brain-collection.rsyd.dk/). A total of 10 samples from 4 patients were included, representing the following anatomical brain regions: pons, thalamus, cerebellar vermis, and corpus striatum. An overview of the 10 FFPE blocks used and their tissue age and identity is shown in **Table 1**. The included patients were identified by searching for "epilepsy" in a database of the original patient diagnoses, and then choosing patients based on information from discharge letters stored on a GDPR-secured server, both provided by the Open Patient data Explorative Network (OPEN), OUH and Department of Clinical Research, University of Southern Denmark, Odense, Denmark. The specific pathological cohort is not the focus of the current study, which is instead intended to develop a methodological framework for the molecular study of aged FFPE blocks stored at the Danish Brain Collection.

Table 1: FFPE brain samples included in this study.

Patient	Sex	Age at death (years)	Year of death	Sample age (years)	ID	Brain tissue	Fixative agent
A	F	68	1952	73	A1	Pons	10% FF
					A2	Corpus striatum	
B	M	52	1964	61	B1	Thalamus	10% FF
					B2	Cerebellar vermis	
					B3	Pons	
					B4	Corpus striatum	
C	F	38	1967	58	C1	Pons	N/A
					C2	Corpus striatum	
D	M	63	1973	52	D1	Cerebellar vermis	Cajal's fixative
					D2	Corpus striatum	

M = male; F = female; FF = formalin fixation; N/A: No information available.

Ethics declaration

The study was approved by The Danish National Committee on Health Research Ethics (ethical

permission journal and supplement no. CVK-2308632 and CVK-112142) in accordance with the

Danish Act on Research Ethics Review of Health Research Projects (Komitéloven) and all

methods were performed in accordance with relevant guidelines and regulations.

The study is based on archival human biological material collected between 1945 and 1982, prior

to the introduction of requirements for obtaining informed consent for the samples in question.

Consequently, individual informed consent has not been obtained. This general issue concerning

The Brain Collection has been addressed previously in 2006 by The Danish National Committee

on Biomedical Research Ethics journal number 2005-7049-6.

Under Danish law, research use of such archival material without consent may be permitted

subject to approval by the competent research ethics committee, cf. Komitéloven. The present

study has been reviewed and approved on this basis.

Preprocessing

FFPE blocks from each patient were selected based on the registered time of death of the

individual, to ensure that a range of different preservation ages were included in the pilot study

to assess any potential effects of storage time on the quality of molecular data. Additionally, FFPE

blocks containing tissue from four different neuron-rich brain areas were included, representing

tissue-specific variation across different brain regions. Tissue identity of each block is provided

in **Table 1.** First, each FFPE block was sectioned into three separate pieces to ensure comparable

cell-rich grey matter content. To remove paraffin from the tissue, two pieces were incubated

overnight at 50°C, then for 1 hour at 60°C. The first piece was left in its melted state (referred to

as "Melted") and stored at -20°C. The second piece was re-embedded in paraffin and not used

any further in this study. The third piece remained unprocessed in its original state (referred to as

"original FFPE") and stored at room temperature in a dry, dark environment before being

transferred to -20°C.

Isolation of DNA and RNA

All laboratory procedures were carried out in clean laboratory facilities specifically dedicated to

the processing of historical and/or ancient samples within the GLOBE Institute (University of

Copenhagen, Denmark). DNA was extracted from FFPE blocks using three approaches: (i) the

DNeasy Blood & Tissue Kit (Qiagen) with heat-mediated decrosslinking at 90°C [16], (ii) the

same kit with an additional deparaffinization step using Deparaffinization Solution (Qiagen), and

(iii) the AllPrep® DNA/RNA FFPE Kit (Qiagen) for simultaneous DNA and RNA recovery. RNA

was independently extracted using the mirVana™ miRNA Isolation Kit (Thermo Fisher

Scientific), selected based on its prior successful application for RNA extraction from ancient

tissue samples [32,34], with additional pretreatment steps including heat-mediated

deparaffinization and decrosslinking. Detailed protocols for each extraction method, including

reagent volumes, incubation conditions, and all modifications to the manufacturers'

recommendations, are provided in **Supplementary Methods**. Additional metadata for DNA and

RNA extracts is also available in **Supplementary Tables 1** and **2**. Nucleic acid concentrations

were measured using Qubit™ High Sensitivity dsDNA and RNA kits (Thermo Fisher Scientific)

with a Qubit v3.0 fluorometer (Thermo Fisher Scientific). Size distribution and integrity of the

DNA and RNA extracts for each sample were assessed using the High Sensitivity RNA

ScreenTape, and High Sensitivity D1000 DNA ScreenTape in an Agilent 2200 TapeStation

System (Agilent Technologies).

Library preparation

DNA libraries were prepared following the Blunt-End-Single-Tube (BEST) protocol [35,36],

comprising end-repair, adapter ligation, and fill-in reaction steps. Libraries were quantified by

qPCR (Mx3005P, Agilent Technologies), indexed with unique dual 8-bp indices, and purified

using HighPrep PCR beads (MagBio Genomics). cDNA libraries from RNA extracts (n = 20,

from mirVana and AllPrep extractions) were prepared using the NEXTFLEX Small RNA-Seq

Kit v4 with Unique Dual Indexes (Revvity), without size selection. For a subset of 10 RNA

samples (from A1, A2, B2, C2, D1 pairs), end-repair was performed prior to additional library

preparation using T4 Polynucleotide Kinase (T4 PNK, NEB) to repair damaged 5' and 3' termini

on fragmented RNA molecules. A detailed protocol for all library preparation steps, including

reagent compositions, thermocycler programs, and clean-up procedures, is provided in

Supplementary Methods. Library concentrations were quantified using the Qubit™ dsDNA

Quantification Assay Kit (Thermo Fisher Scientific) with a Qubit 2.0 Fluorometer (Thermo Fisher

Scientific). The size distribution of the DNA and cDNA libraries was measured using the High

Sensitivity DNA assay (Agilent Technologies) with a Bioanalyzer 2100 system (Agilent

Technologies).

Sequencing

The DNA (n = 33) and cDNA (n = 30) libraries were separately submitted to Novogene (Planegg,

Germany), and independently sequenced on an Illumina NovaSeq X Plus system, with a full 10B

lane in 150 bp paired-end configuration. For cDNA libraries, a homogeneous distribution of

sequencing effort across all samples was applied. For DNA libraries, we balanced the sequencing

effort so that each of the four original individuals obtained equivalent amounts of sequences at a

target genome-wide coverage of at least 10×.

Statistical analysis

Statistical comparison of DNA and RNA yield between extraction methods and pretreatment

conditions was performed using two-sided paired t-tests in R v4.4.2. *P*-values are reported without

correction for multiple testing.

Sequence analysis

Raw FASTQ files were quality-filtered using fastp v0.24.1 [37] with the following parameters:

automatic adapter detection and trimming, polyG tail removal, paired-end read merging, a

minimum sequence length of 30 nt for DNA and 18 nt for RNA data, and a minimum overlap of

30 and 15 matching nucleotides for paired-end merging of DNA and RNA sequences,

respectively. Only successfully merged paired sequences were retained for subsequent analyses.

The human RefSeq assembly (GRCh38.p14, GCF_000001405.40) and associated gene

annotations were used as reference. A reference transcriptome was generated using gffread v0.12.6 [38], retaining only the longest transcript isoform per gene across protein-coding messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), long noncoding RNAs (lncRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs). The microRNA (miRNA) annotation was retrieved from miRGeneDB v3.0 [39].

Alignment was performed using Bowtie2 v2.4.2 [40] with end-to-end sensitive mode. DNA sequences were first sorted with the sambamba v0.8.0 tool [41], then deduplicated using the markdup function from SAMtools v.1.10 [42]. RNA sequences were not deduplicated, as the absence of unique molecular identifiers (UMIs) in the library preparation protocol prevents reliable distinction between PCR duplicates and genuinely identical RNA fragments derived from highly expressed loci. RNA library complexity was instead estimated as 1 minus the duplication rate reported by fastp. Genic and intergenic mapping was resolved using BEDtools v2.31.0 [43]. RNA sequences mapped to the reference transcriptome that were also identified as either overlapping intergenic or intronic regions genome-wide were flagged and removed. Taxonomic classification of DNA and RNA sequences was performed using KrakenUniq v0.7.3 [44] with a custom database [45] to characterize endogenous human content and exogenous contamination. Additional details are described in **Supplementary Methods**.

Gene expression quantification

RNA sequences mapped to the human reference transcriptome were assigned to each corresponding gene with the intersect function from BEDtools v2.31.0 [43], and the percentage of the transcript body supported by any mapped read (breadth of coverage) was determined with the BEDtools coverage function. For miRNA genes, RNA sequences mapped to mature miRNA loci were considered as successful alignments if they overlapped the corresponding region by at least 75% of their length (bedtools intersect -f 0.75). Genes were considered successfully captured if they had a transcript-wide coverage >10%.

Damage analysis

The PMDtools v0.60 [46] software was used to identify C>U deamination events in both RNA

and DNA sequencing data. For DNA data, PCR-deduplicated sequences mapped to the GRCh38

assembly were analyzed over the first 50 nucleotides of each mapped sequence. For RNA,

sequences mapped to the custom transcriptome were assessed over 30 nucleotides with single-

stranded mode enabled. Only C>U deaminated sites, read as C>T by the sequencer, were

considered.

Clustering and tissue-specific gene expression analysis

Protein-coding mRNA and miRNA quantification for each RNA sample were used as input for

Principal Component Analysis (PCA) after variance-stabilizing transformation with DESeq2

v1.44.0 [47]. For tissue-specific clustering, UMAP dimensionality reduction was applied using

the umap v0.2.10.0 R package (https://github.com/tkonopka/umap). As modern reference, we

used a comprehensive human RNA-seq atlas retrieved with the getAtlasExperiment function (E-

MTAB-2836) from ExpressionAtlas v1.32.0 R package [48] including 11 healthy tissues [49,50],

supplemented with chondrocyte samples [51] and four brain regions [52]. For the microRNA

fraction, small RNA-seq datasets from human healthy tissues were used [52–54]. A

comprehensive list of all reference samples is provided in **Supplementary Table 3**.

Quantification values were normalized with the trimmed mean of M-values (TMM) method [55]

using edgeR v4.2.1 [56] and $\log_2$ transformed. FFPE RNA data were then projected onto the

learned UMAP embedding. Further details on reference data processing and UMAP parameters

are provided in **Supplementary Methods**.

Hierarchical clustering was performed separately on protein-coding mRNA and miRNA profiles

from end-repaired libraries (n = 10) using the top 100 most variable genes. TMM-normalized

$\log_2$-transformed CPM values were first converted to z-scores per gene and clustered with the

Ward's minimum variance method (ward.D2) and Euclidean distance, then visualized using

ComplexHeatmap v2.20.0 [57]. Tissue specificity was quantified using the tau (τ) index [58],

computed from the reference multi-tissue atlas. The tau index was defined as:

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$$\tau = \frac{\sum_{i=1}^{n}(1-\hat{x}_i)}{n-1}; \quad \hat{x}_l = \frac{x_i}{\max(x)}$$

where n is the number of tissues, $\hat{x}_i$ is the expression normalized to the tissue with maximum

expression, and x_i is the average expression in tissue i. Tau values range from 0 (uniform

expression) to 1 (tissue-restricted). Validated tissue markers were defined as genes with: (i) $\tau \geq$

0.5; (ii) peak expression in cerebellar vermis or corpus striatum in the reference atlas; (iii)

detectable FFPE abundance (average $\log_2$ CPM ≥ 3); (iv) transcript coverage > 10% in the

corresponding FFPE tissue (protein-coding genes only); and (v) concordant directionality

between the reference atlas and FFPE data. Full details on clustering and filtering criteria, as well

as on gene selection are provided in **Supplementary Methods.**

Results

We have analyzed post-mortem formalin-fixed paraffin-embedded (FFPE) brain tissue from the

Danish Brain Collection, obtained from autopsies carried out at Danish state hospitals between

1945 and 1982 (**Table 1**). Both DNA and RNA were extracted in bulk, sequenced and analyzed

comparing different laboratory approaches for pretreatment, nucleic acid isolation and

transcriptional damage repair. Complete metadata information including pretreatment, extraction

batch, sample identity, extraction method and nucleic acid yield is available in **Supplementary**

Table 1 for DNA and **Supplementary Table 2** for RNA extracts.

Pretreatment of FFPE blocks and nucleic acid extraction method affect DNA yield

We first sought to compare the effectiveness of different methods for removing paraffin from

FFPE brain tissues, and how this impacted the DNA concentration obtained after nucleic acid

extraction. Overnight paraffin melting significantly increased DNA yield compared to original

untreated FFPE samples without paraffin removal (P-value = 1.8×10^{-4}), and this was also

observed when applying a deparaffinization solution (**Fig. 1A**), although it did not reach statistical

significance (*P*-value = 0.1752). All samples tested for pretreatment conditions and their DNA

yield are in **Supplementary Table 4**.

Figure 1: Pretreatment and extraction protocol impact on DNA and RNA yield. (**A**) DNA

yield obtained from FFPE samples B1, B2, and B4 using two pretreatment approaches: physical

removal of excess paraffin (original vs. melted samples) and enzymatic deparaffinization (see

Methods). (**B**) Fold change of the difference in DNA yield between the AllPrep and Blood &

Tissue methods for DNA extraction. A positive fold change value means a higher DNA yield was

obtained with the AllPrep protocol, and vice versa. (**C**) Bland-Altman plot on the agreement

between AllPrep and mirVana methods for RNA extraction according to the yield obtained with

both protocols. The dark red dotted line represents the average difference in RNA yield in $\log_2$

scale. Light grey dotted lines represent the upper and lower confidence intervals at ±1.96 standard

deviations from the average. (**D**) Bland-Altman plot on the agreement between AllPrep and

mirVana methods for RNA extraction according to the RNA integrity (RIN) values obtained with

both protocols. The dark red dotted line represents the average difference in RIN values. Light

grey dotted lines represent the upper and lower confidence intervals at ±1.96 standard deviations

from the average. Additional data from patients A, B, C, and D can be found in **Table 1**.

Next, we compared two different DNA extraction approaches on FFPE blocks. The DNeasy

Blood & Tissue method from Qiagen is designed for simple and efficient recovery of DNA from

different types of tissues. Incidentally, it can also recover RNA molecules during the extraction

protocol [16], but we did not use this fraction from this particular kit. Moreover, it is not

specifically tailored for FFPE samples, so we adapted it to include an initial decrosslinking step

following prior reports demonstrating the efficacy of heat-mediated decrosslinking for FFPE

DNA recovery [16]. In contrast, the other method we tested (AllPrep® DNA/RNA FFPE also from

Qiagen) is specifically designed to work with FFPE tissues and allows simultaneous isolation of

DNA and RNA molecules from the same sample. Despite this, it is a more time-consuming and

involved protocol requiring more than double the time for its manual implementation. We again

extracted DNA in an independent batch from all ten FFPE brain samples using both approaches.

Upon comparison of both extraction kits, we found differences in both DNA yield and fragment

length distribution, with an overall higher but variable DNA yield obtained with the AllPrep

protocol compared to the Blood & Tissue protocol (**Fig. 1B**). Numeric values for total DNA

concentration in all analyzed samples of this batch are available in **Supplementary Table 5**.

Extraction protocol affects RNA yield but not RNA integrity

Next, to determine the efficacy of RNA isolation from decades-old FFPE tissues, we tested two

different kits and evaluated both RNA yield and transcript integrity (as expressed by RIN values).

We again compared the AllPrep kit, which allows RNA extraction jointly with DNA, and the

mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific), which is optimized for short-length

RNA recovery. The extraction method significantly influenced RNA yield, but not the RNA

integrity. The use of the AllPrep kit resulted in extracts with significantly higher RNA

concentration than the mirVana protocol (P-value = 3.79×10^{-3}). Interestingly, the AllPrep

method showed a higher tendency to improve the RNA yield over the mirVana kit at lower

concentrations (**Fig. 1C**). Conversely, there were no significant differences in RIN values between

the mirVana and AllPrep techniques (**Fig. 1D**, *P*-value = 0.2197), and the AllPrep kit only

marginally improved the RNA integrity obtained at RIN values >2. Numeric values for total RNA

concentration and RIN values in all analyzed samples are available in **Supplementary Table 6**.

Decades-old FFPE brain samples yield mappable nucleic acids

After sequencing and mapping to the human (GRCh38) genome assembly, both DNA and RNA

from FFPE brain tissues in the DBC showed a high alignment rate, with overall above 75% of

sequences successfully mapped (**Supplementary Table 7**, **Figs. 2A** and **2B**). However, DNA

sequences belonging to patient D had ultra-low successful mapping, with only about 1.5% of them

mapped to the human assembly (**Supplementary Table 7**, **Fig. 2A**). The remaining unmapped

sequences in patient D libraries were attributable to a combination of microbial DNA consistent

with environmental contamination (**Supplementary Table 8**, see Methods), library artifacts, and

unmappable ultrashort fragments resulting from severe DNA degradation. These observations,

combined with high duplication rates (**Supplementary Table 7**) indicate severe DNA

degradation in these FFPE blocks, likely reflecting variation in historical fixation conditions

within the collection as described in **Table 1**.

Consistent with this observation, samples from patients A, B, and C clearly outperformed those

from patient D not only in the number of mapped DNA sequences, but also in library complexity

(**Fig. 2C**), i.e. the number of uniquely aligned sequences after deduplication. Moreover, the

fragment length distribution of successfully mapped DNA sequences was markedly shifted

toward shorter fragments in samples from patients A and D compared to patients B and C, which

showed longer and more dispersed fragment lengths with an average of ~60 nucleotides (**Fig.**

2D). Interestingly, patient D, despite being the most recently deceased of the four individuals

(date of death recorded in 1973, **Table 1**), showed shortened DNA fragments mapped to the

human assembly, similar to the profiles observed for patient A, the oldest of the four (**Fig. 2D**).

This, together with the very low number of mapped DNA sequences and extensive degradation

of DNA in the FFPE blocks from this individual, suggests that chemical treatment during fixation,

rather than age, is the main driver of DNA quality observed in our data.

Figure 2: Sequencing and mapping statistics of DNA and RNA libraries from decades-old FFPE brain samples. (**A**) Percentage of DNA sequences successfully mapped to the human genome assembly (GRCh38) per library (see **Table 1** and **Supplementary Table 1** for additional data). (**B**) Percentage of RNA sequences successfully mapped to the human genome assembly (GRCh38) per library (see **Table 1** and **Supplementary Table 2** for additional data). (**C**) Library complexity of DNA samples, defined as the percentage of uniquely mapped sequences after deduplication. Fragment length density distribution of (**D**) DNA and (**E**) RNA sequences mapped to the human genome, grouped by patient and the recorded date of death highlighting the age of the FFPE blocks. (**F**) Proportion of mapped sequences overlapping exonic, intronic, and

intergenic regions for DNA and RNA samples. Grey dashed values represent the proportion of exonic, intronic and intergenic regions annotated genome-wide in the human assembly (GRCh38). See Methods for more details on the loci considered. (**G**) RNA biotype composition of sequences mapped to exonic regions, showing the relative proportion of ribosomal RNA (rRNA), transfer RNA (tRNA), protein-coding messenger RNA (mRNA), long-noncoding RNA (lncRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) annotated loci. (**H**) Library complexity of RNA samples processed with and without T4 PNK end-repair prior to library preparation.

These patterns contrast with those observed for the mapped RNA sequences, where most fragments were of ultrashort length, between 18 and 30 nucleotides (**Fig. 2E**). As with DNA samples, no clear age-related differences in RNA fragment length distribution were observed. Interestingly, contrary to what was observed in DNA, FFPE RNA from patient D samples showed profiles similar to the rest (**Supplementary Table 7**), including scarce microbial RNA contamination (**Supplementary Table 9**). Nevertheless, despite their short overall fragment length, RNA sequences showed clear signs of transcriptome-like mapping patterns, with nearly 35% of mapped fragments falling within annotated exonic regions of the human genome assembly (GRCh38, **Fig. 2F, Supplementary Table 7**). Intronic and intergenic mapping RNAs accounted for approximately 22% and 44% of the sequences, respectively. Conversely, DNA sequences showed markedly different proportions, more closely reflecting the overall genomic distribution of exonic, intronic, and intergenic regions, where DNA fragments mapped to exonic regions accounted for less than 2% of the total mapped sequences (**Fig. 2F**). Among exonic RNA sequences, the vast majority overlapped with rRNA loci, followed by tRNA, and protein-coding loci (**Fig. 2G**). Other less abundant noncoding loci such as lncRNAs, snRNAs and snoRNAs were also detected (**Fig. 2G**). Comprehensive abundance and transcript coverage profiles for all transcript types analyzed (see Methods) are available in **Supplementary Tables 10-15.**

End-repair treatment increases library complexity but shortens RNA fragments

We next evaluated the effect of end-repairing damaged RNA fragment termini from decades-old

FFPE samples on adapter ligation efficiency during library preparation, and its impact on the

resulting transcriptomic sequencing data. To do so, we processed 10 samples (**Supplementary**

Table 2) with and without applying T4 PNK end-repair prior to library preparation (see Methods).

All end-repaired samples showed a relative increase in library complexity (**Fig. 2H**), defined as

the fraction of non-redundant (i.e. non-PCR-duplicate) RNA fragments. Samples without end-

repair treatment had an average 57% complexity, meaning that over 40% of sequencing effort

likely consisted of redundant PCR duplicates, while end-repaired samples showed an average

71% complexity (**Fig. 2H**).

Consistent with the higher library complexity observed for RNA samples, end-repair also

significantly increased the number of protein-coding mRNA loci profiled at >10% transcript

coverage (**Figs. 3A** and **3B**), a threshold we used as a heuristic for reliably detected genes [32].

This effect was observed across all 10 samples evaluated with and without end-repair treatment

(**Supplementary Figs. 1**-**2**). Furthermore, for some of the more degraded and poorly profiled

samples, particularly those from patients A and C, end-repair was essential to recover any protein-

coding mRNA genes above this coverage threshold (**Supplementary Figs. 1**-**2**). Interestingly,

several highly abundant mRNA loci detected in samples without end-repair (**Fig. 3A**), although

at ultra-low coverage (<10%), showed reduced abundance but increased transcript coverage after

end-repair (**Fig. 3B**). This pattern suggests that end-repair enables a more even sampling of

mRNA fragments across transcripts, rather than libraries being dominated by PCR duplicates of

a small number of highly abundant loci. However, the increased resolution obtained for the

protein-coding fraction in end-repaired RNA libraries did not extend to microRNA loci. Libraries

without end-repair treatment yielded a higher number of profiled microRNA genes (**Fig. 3C**) and

more mapped RNA fragments overlapping miRNA loci compared to end-repaired samples (**Fig.**

3D), suggesting that end-repair is detrimental for microRNA recovery. A comprehensive

quantification of all microRNAs detected is available in **Supplementary Table 16**.

End-repaired samples also showed a reduced average RNA fragment length compared to samples

without end-repair, an effect that was consistent across all gene types analyzed but particularly

pronounced for the most abundant transcript classes: rRNAs, tRNAs, and protein-coding mRNAs

(**Fig. 3E**).

Figure 3: End-repair effects on RNA library composition and tissue-specific gene expression

profiling from FFPE brain samples. (**A**) Sequence count vs. transcript coverage for protein-

coding mRNAs in the 2A library from patient B without end-repair treatment (B2A sample). The

red dashed line marks a 10% coverage threshold, defined as the lower limit to consider a given

protein-coding mRNA gene as successfully profiled. (**B**) Sequence count vs. transcript coverage

for protein-coding mRNAs in the 2A library from patient B with end-repair treatment (B2AER

sample). (**C**) Number of microRNA loci profiled per sample, comparing libraries with and without

end-repair treatment. (**D**) Number of mapped microRNA sequences per sample, comparing

libraries with and without end-repair treatment. (**E**) RNA fragment length density distribution for

protein-coding mRNA, rRNA, and tRNA genes, comparing end-repaired (red) and non-end-

repaired (grey) libraries. (**F**) PCA of protein-coding mRNA expression profiles across all 30 FFPE

brain samples. The left panel shows all samples colored by end-repair status. The right panel

shows the same data after regressing out the end-repair effect, with samples colored by brain

region. (**G**) PCA of microRNA expression profiles across all 30 FFPE brain samples. The left

panel shows all samples colored by end-repair status. The right panel shows the same data with

samples colored by brain region. (**H**) UMAP embedding of protein-coding mRNA expression

from end-repaired FFPE brain samples (n = 10) projected onto a human multi-tissue RNAseq

reference atlas. (**I**) UMAP embedding of microRNA expression from end-repaired FFPE brain

samples projected onto a human multi-tissue small RNAseq reference atlas. FFPE sample pairs

per tissue belong to patients A (n = 4, A1: Pons & A2: Corpus striatum), B (n = 2, B2: Cerebellar

vermis), C (n = 2, C2: Corpus striatum), and D (n = 2, D1: Cerebellar vermis). The hindbrain

region comprises pons and cerebellar vermis tissues, while the forebrain region comprises corpus

striatum tissue.

Damage profiles indicate moderate cytosine deamination in FFPE-derived DNA and RNA

To assess the extent of chemical damage in the sequenced nucleic acids, we examined nucleotide

misincorporation patterns along mapped DNA and RNA fragments. For DNA, all samples

showed a characteristic cytosine-to-uracil (C>U) mismatch enrichment at the 5' end of fragments

and the complementary guanine-to-adenine (G>A) enrichment at the 3' end (**Supplementary Fig.**

3), consistent with cytosine deamination induced by formalin fixation and post-mortem hydrolytic

damage, as typically observed in degraded and archival DNA [10,59,60]. The magnitude of these

terminal misincorporation frequencies was moderate, reaching approximately 2-4% at the first

position across all samples, with a gradual decay toward background levels within the first 5-10

nucleotides. This pattern was consistent across all four patients regardless of sample age. For

RNA samples, cytosine deamination frequencies were assessed along end-repaired fragments

(**Supplementary Fig. 4**). Average deamination rates ranged from less than 1% to ~3.5%, with a

slight enrichment at the 5' terminal positions relative to the fragment body. Compared to the DNA

profiles, RNA deamination rates were of similar or slightly higher magnitude but showed a more

uniform distribution along the fragment length rather than a steep terminal enrichment, consistent

with the combined effects of formalin-induced and hydrolytic cytosine deamination acting across

the full length of single-stranded RNA molecules. Samples from patients A and D showed higher

deamination rates compared to those from patients B and C (**Supplementary Fig. 4**), broadly

consistent with the increased DNA fragmentation observed for these individuals (**Fig. 2D**).

Decades-old FFPE brain samples retain tissue-specific gene expression signatures

To determine whether the transcriptomic data recovered from FFPE brain samples captured

biologically meaningful gene expression patterns, we performed dimensionality reduction

analyses on both protein-coding mRNA and microRNA quantification profiles. PCA on protein-

coding mRNA expression confirmed that end-repair status was the dominant source of variance,

accounting for 56.25% of the total variation along PC1 (**Fig. 3F**, left panel). After regressing out

the end-repair effect, the corrected PCA (**Fig. 3F**, right panel) revealed a clear separation by brain

region along the first two components, with cerebellar vermis samples clustering distinctly from

pons, corpus striatum, and thalamus samples. For microRNAs, PCA showed a less structured

pattern (**Fig. 3G**, left panel) when examining the end-repair status, but separation by brain region

became apparent when examining PC2 vs. PC3 (**Fig. 3G**, right panel), again with cerebellar

vermis as the most distinct tissue. No clear influence of sex was observed for either protein-coding

mRNAs or microRNAs (**Supplementary Figs. 5A** and **5B**).

To place these expression profiles in a broader biological context, we first projected the FFPE

brain protein-coding mRNA profiles from end-repaired samples onto a human multi-tissue

reference atlas (**Fig. 3H**). The FFPE brain samples consistently clustered adjacent to reference

brain tissue samples, distinct from all other tissues included in the reference atlas, including

skeletal muscle, heart, liver, lung, kidney, skin, and gut-derived tissues, among others. Moreover,

we found a clear divergence between end-repaired FFPE samples obtained from cerebellar vermis

tissue compared to those from the corpus striatum (**Fig. 3H**). Samples from patient A (Pons tissue)

did not cluster with other hindbrain-derived tissues such as the cerebellar vermis, nor did they

form a separate cluster. This result confirms that despite decades of formalin fixation and paraffin

embedding, these archival brain samples retain gene expression signatures that are recognizably

brain-specific, reflecting differentiated protein-coding transcriptional programs between

forebrain and hindbrain regions in the central nervous system. A similar pattern was found when

projecting FFPE samples without end-repair treatment onto the same multi-tissue protein-coding

human reference atlas, with all samples clustering nearby or adjacent to brain-derived tissues

(**Supplementary Fig. 5C**). However, only those obtained from cerebellar vermis tissue showed

a clear tissue-specific clustering pattern.

For microRNA profiles, projection onto a multi-tissue human microRNA expression atlas also

showed a clearly defined clustering pattern, with end-repaired FFPE brain samples dividing into

those derived from the hindbrain (pons and cerebellar vermis), and those derived from the

forebrain (corpus striatum), again reflecting a shared post-transcriptional regulatory program

among brain tissues of common developmental origin (**Fig. 3I**). When considering FFPE samples

without end-repair treatment, however, such division was not apparent, although all of them

clustered adjacent to brain-derived tissues included in the microRNA expression reference

(**Supplementary Fig. 5D**).

Unsupervised hierarchical clustering of the top 100 most variable protein-coding mRNA genes

separated the 10 FFPE samples primarily by patient and then by brain region, with cerebellar

vermis and corpus striatum samples forming two distinct clusters (**Supplementary Fig. 6**). The

two pons samples, both derived from patient A, clustered separately and were not further

evaluated as region-specific. A similar tissue-driven separation was observed for the top 100 most

variable miRNAs (**Supplementary Fig. 7**).

Furthermore, to determine whether this clustering reflects known regional gene expression

patterns, we computed tissue specificity values (τ index) for all protein-coding mRNA and

microRNA genes included in the multi-tissue gene expression collections used as reference

(**Supplementary Tables 17-18**). Filtering criteria included tissue-restricted expression ($\tau \geq 0.5$),

concordant directionality between the reference atlas and FFPE end-repaired samples, detectable

abundance in FFPE samples, and adequate transcriptional coverage (see Methods). Across the

full transcriptome, 321 protein-coding genes were validated as tissue markers: 177 in cerebellar

vermis and 144 in corpus striatum. Of these, 28 fell within the top 100 most variable genes in the

hierarchical clustering (18 cerebellar vermis, and 10 corpus striatum; **Supplementary Table 19**).

Among the validated cerebellar vermis markers, several canonical cerebellar genes were

identified, including the granule cell-associated zinc finger transcription factors *ZIC1* and *ZIC4*,

the Purkinje cell synapse organizers *CBLN1* and *CBLN3*, the metabotropic glutamate receptor

GRM1, the ETS transcription factor *ETV1*, and the voltage-gated potassium channel *KCNA1*,

among others. Additional well-established cerebellar markers such as *GABRA6*, *PVALB* and

PAX6 were validated outside the top 100 variable gene set (**Supplementary Table 19**). Corpus

striatum-enriched markers included the neuropeptide *CCK*, the postsynaptic calmodulin-binding

protein *NRGN*, the calcium/calmodulin-dependent kinase *CAMK2A*, the synaptic protein *DDN*,

and the cortexin *CTXN1*. The neuropeptides *SST* and *NPY* were among the strongest striatal

markers validated outside the top 100 (**Supplementary Table 19**).

For miRNAs, 14 were validated as tissue-specific markers in end-repaired FFPE samples, of

which 13 were among the 100 most variable according to hierarchical clustering (**Supplementary**

Table 20). Four were detected as predominant in cerebellar vermis and ten in corpus striatum.

The strongest cerebellar miRNA markers were miR-124 paralogues (miR-124-P1-v1 and miR-

124-P2-v2), along with miR-376-P2 and miR-154-P14. Corpus striatum-related miRNAs

included miR-137 (τ = 0.97), as well as miR-138-P2, miR-132, and miR-433 (**Supplementary**

Table 20).

Discussion

In this study, we demonstrate that decades-old FFPE brain tissue from the Danish Brain Collection

retains sufficient molecular integrity for informative genomic and transcriptomic analyses.

Systematic evaluation of extraction, library preparation, and bioinformatic approaches shows that

archival samples yield recoverable DNA and RNA with sequencing data capturing biologically

meaningful, tissue-specific expression patterns.

Practical considerations for nucleic acid recovery from aged FFPE tissues

Comparison of extraction methods identified the AllPrep protocol as the most suitable for DBC

material, yielding higher DNA and RNA concentrations than Blood & Tissue and mirVana while

requiring less input tissue. Its ability to simultaneously recover DNA and RNA from the same

specimen is particularly advantageous for limited archival material and enables parallel genomic

and transcriptomic analyses.

We note, however, that the Blood & Tissue protocol for DNA extraction yielded longer fragments,

indicating a trade-off between yield and fragment length. While longer DNA sequences may

benefit applications such as haplotype phasing or structural variant detection, the higher yield

from AllPrep was more advantageous for the short-read sequencing used here.

Fixation chemistry drives variability in nucleic acid preservation

Not all samples performed comparably. Tissues from patient D exhibited markedly reduced DNA

mapping rates and library complexity relative to the rest of the cohort. Notably, these samples

were preserved using Cajal's fixative rather than neutral buffered formalin. Cajal's fixative is

highly acidic (pH ~1.5) [61], a condition known to accelerate depurination and phosphodiester

bond cleavage in nucleic acids [25]. Consistent with prior reports, acidic or unbuffered fixation

leads to increased fragmentation and reduced amplifiability compared to neutral conditions

[16,28]. Therefore, the poor performance of patient D samples might be explained by the fixation

chemistry rather than by tissue age, given that patient D is paradoxically the most recently

deceased individual in the cohort (date of death 1973).

Patient A, the oldest individual in the cohort (1952), exhibited the shortest DNA fragment lengths

and reduced RNA library complexity. The two available pons samples clustered with other brain

regions but were insufficient (n = 2, single individual) for robust assessment of region-specific

gene expression. Whether this reflects intrinsic properties of the pons, patient-specific

degradation, or both cannot be resolved with the current data. Together with the findings from

patient D, these results indicate that fixation chemistry, rather than specimen age, is the dominant

determinant of nucleic acid preservation in archival material. Accordingly, detailed metadata on

fixation conditions (i.e. fixative type, concentration, pH, duration, and temperature) should be

systematically recorded and incorporated into study design, sample selection, and downstream

analyses when working with FFPE collections.

End-repair improves FFPE transcriptomics

The application of T4 PNK end-repair prior to library preparation emerged as a key step for

recovering informative transcriptomic data from highly degraded FFPE RNA. End-repaired

libraries showed substantially higher complexity, with a greater proportion of sequences

representing independent RNA molecules rather than PCR duplicates. This improvement

translated into enhanced gene detection sensitivity, enabling the tissue-specific gene expression

profiling reported here.

The effect of T4 PNK end-repair likely reflects restoration of damaged 5' and 3' termini on

fragmented RNA, which in their unrepaired state cannot efficiently ligate to sequencing adapters

during library preparation. This is consistent with reports demonstrating improved mRNA capture

from extracellular vesicles and plasma RNA [62,63], and extends these findings to the more

severely degraded context of decades-old FFPE tissue. A trade-off is a relative reduction in

microRNA representation, as the increased capture of longer repairable RNA fragments likely

dilutes the small RNA fraction in the final library. This is an important consideration for study

design, as omitting end-repair may be preferable when small RNAs are the primary target.

Nevertheless, end-repaired libraries retained biologically informative miRNA profiles that

recapitulated tissue-specific patterns observed in untreated samples, and more clearly resolved

the hindbrain-forebrain distinction, consistent with differences in embryological origin and post-

transcriptional regulation.

Furthermore, the shorter fragment length distribution observed in end-repaired libraries likely

reflects either preferential restoration of termini on shorter damaged fragments, rendering them

ligatable and thereby shifting the overall distribution, additional fragmentation introduced during

enzymatic treatment and heat inactivation, or a combination of both. Future optimization could

explore alternative inactivation strategies, such as bead-based cleanup without heat exposure, to

preserve ultrashort fragments while retaining the gains in library complexity conferred by end-

repair.

Biological validity of FFPE-derived transcript abundance profiles

A central finding of this study is that archival FFPE brain tissue retains recognizably tissue-

specific transcriptomic signatures despite decades of formalin fixation and paraffin embedding.

When projected onto independent modern multi-tissue reference atlases, FFPE brain samples

clustered with brain-derived tissues and were clearly separated from non-neural tissues, across

both protein-coding mRNA and microRNA profiles. Within the brain compartment, cerebellar

vermis and corpus striatum were readily distinguished, recapitulating the well-documented

transcriptomic divergence between hindbrain and forebrain regions [64–66].

Tissue specificity analysis using the tau index provided quantitative support for these

observations. Of the 321 protein-coding genes and 14 microRNAs validated as tissue markers,

many are canonical regional markers with well-characterized roles in cerebellar or striatal

biology. Cerebellar vermis markers included the zinc finger transcription factors *ZIC1* and *ZIC4*,

which define granule cell identity [67,68], the synaptic organizers *CBLN1* and *CBLN3*, essential

for Purkinje cell synapse formation [69–71], the GABA-A receptor subunit *GABRA6*, exclusively

expressed in cerebellar granule cells [72–74], and *PVALB*, a calcium-binding protein enriched in

Purkinje cells [75]. In the corpus striatum, validated markers included the interneuron-associated

neuropeptides *SST* and *NPY* [76,77], the neuron-specific kinase *CAMK2A* [78,79], and the

calcium/calmodulin-dependent kinase *CAMKV* [80].

MicroRNA markers were similarly consistent with known regional biology. MiR-137, a robustly

forebrain-enriched microRNA and a GWAS locus for schizophrenia [81,82], was among the

strongest striatal markers, while the miR-124 family showed the expected cerebellar

predominance [83–86]. The recovery of these well-established markers from tissue dating to the

1940s and 1950s demonstrates that regional molecular identity can survive the combined effects

of post-mortem interval, formalin fixation, and prolonged storage.

Not all established markers met our validation criteria. Several canonical striatal genes, including

HTR2C [87], *DRD2* [88], and *GNAL* [89], were detectable in FFPE corpus striatum samples but

did not achieve sufficient transcriptional coverage for robust analysis. This highlights a key

limitation of FFPE-based transcriptomics: while transcript presence and relative abundance can

be captured, a comprehensive transcriptional reconstruction remains challenging for a subset of

loci. Broader sampling across FFPE blocks and brain regions may improve transcript recovery,

but further methodological advances will be needed to achieve more complete transcriptome

profiling from highly degraded archival material.

Implications for the Danish Brain Collection

Our results establish a practical framework for molecular profiling of the Danish Brain Collection

and, by extension, similar historical FFPE archives. The combination of AllPrep extraction, T4

PNK end-repair, and single-stranded library preparation enabled recovery of tissue-specific

expression profiles from samples up to 73 years old at the time of analysis. In parallel, a

bioinformatic pipeline adapted from ancient and museum specimen studies [32,34] effectively

accommodated the extensive fragmentation and chemical damage characteristic of these

materials.

The Danish Brain Collection, comprising approximately 9,500 brains spanning four decades of psychiatric history, represents a unique resource for questions inaccessible to contemporary biobanks. These include the molecular correlates of historical diagnostic categories, the impact of changing environmental exposures and treatment regimes on brain molecular profiles, and retrospective investigation of infectious contributors to psychiatric disease in the pre-antibiotic and early antibiotic eras. The metagenomic and metatranscriptomic classification framework applied here, used primarily to assess contamination and library quality, provides a foundation for such investigations and could be extended to systematically screen for pathogen-derived nucleic acids across larger sample sets within the collection. The feasibility of recovering informative genomic and, critically, transcriptomic data from this pilot study opens the door to larger-scale investigations targeting defined diagnostic cohorts within the archive.

Nevertheless, several limitations warrant consideration. First, the sample size reported here is modest (four patients, 10 tissue blocks). Second, tissue representation is uneven across brain regions and individuals due to archival constraints, and the lack of matched fresh-frozen controls prevents direct quantification of information loss associated with FFPE processing. Third, DNA sequencing depth, while sufficient to assess mapping efficiency and damage patterns, would need to be increased to support robust variant calling or comprehensive genomic analyses. These limitations can be addressed in future studies through expanded cohorts, deeper sequencing, and inclusion of matched contemporary controls where available.

Conclusions

In summary, we demonstrate that decades-old FFPE brain tissue from the Danish Brain Collection is amenable to high-throughput DNA and RNA sequencing, retaining biologically meaningful, tissue-specific molecular signatures. These results establish the DBC as a viable resource for retrospective genomic and transcriptomic studies of neurological, neurodegenerative, and psychiatric disorders.

Declarations

Consent for publication

Not applicable

Availability of data and materials

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EM-S and CK conceived this study and devised the experimental design. EM-S and CK secured funding. NO performed metagenomic and metatranscriptomic analyses. CRC and EHF performed laboratory work with support and supervision from SSTM and EM-S. CRC, EHF, EM-S, and NO analyzed the data, with input from CK and MTPG. CRC, EHF and EM-S visualized the data. MW

714 facilitated ethics approval and sample acquisition. CRC, EHF, CK and EM-S wrote the

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