A novel approach to generate enzyme-free single cell suspensions from archived tissues for miRNA sequencing

Stefan Scheuermann a,1, Sarah Hücke b,1, Annika Engel c, Nicole Ludwig d, Philipp Lebhardt a, Jens Langejürgen a, Stefan Kirsch b,*.  

Abstract

Obtaining high-quality omics data at the single-cell level from archived human tissue samples is crucial for gaining insights into cellular heterogeneity and pushing the field of personalized medicine forward. In this technical brief, we present a comprehensive methodological framework for the efficient enzyme-free preparation of tissue-derived single cell suspensions and their conversion into single-cell miRNA sequencing libraries. The resulting data from this study have the potential to deepen our understanding of miRNA expression at the single-cell level and its relevance in the context of the examined tissues. The workflow encompasses tissue collection, RNA later immersion, storage, thawing, Tissue Grinder-mediated dissociation, miRNA lysis, library preparation, sequencing, and data analysis. Quality control measures ensure reliable miRNA data, with specific attention to sample quality. The UMAP analysis reveals tissue-specific cell clustering, while miRNA diversity reflects tissue variations. The presented workflow effectively processes preserved tissues, extending opportunities for retrospective analysis and biobank utilization.

1. Introduction

In the era of personalized medicine, the molecular characterization of diseases is increasing in focus, as the response of numerous therapies depends on the molecular genetic alterations of the individual disease [1]. In particular, addressing diseases with intricate and diverse patterns demands a thorough comprehension of intracellular processes to guide effective treatment strategies [2,3]. Single cell micro RNAs (miRNA) profiling can offer a valuable avenue for deciphering the complexities of these intracellular processes in order to enhance the understanding of individual disease [4]. miRNA are a type of small RNA molecules that play important roles in gene regulation [5]. They show tissue-specific expression patterns and are often dysregulated in diseases, making them valuable biomarkers. In the context of disease treatment, miRNA data can be used to: Identify new biomarkers for diagnosis and prognosis, study the effects of treatments on miRNA expression and develop new targeted therapies that target miRNAs [6–8].

In the last decade, several technologies for single-cell profiling have been developed and used in various applications, revealing many new insights. High-throughput methods for single cell analysis such as flow or mass cytometry and single cell sequencing are ideal for comprehensive identification of single cells based on molecular information [9,10]. These methods have already begun to transform the understanding of complex tissues by enabling the identification of previously unknown cell types and states [11,12]. While single cell sequencing is clearly a powerful diagnostic method, there can be confounding factors in the tissue dissociation process into individual cells that can negatively impact the quality and reliability of the data. One factor is the lack of standardisation, which can lead to significant differences between different research groups and tissue types. Another major challenge is that incomplete disaggregation could bias results towards cell types that are easier to dissociate [13]. A recent study by Wu et al., in which single-cell RNA sequencing was performed using samples from mouse
kidneys, found that endothelial cells and mesangial cells were under-represented in the scRNA-seq data [14]. Ultimately, long enzymatic digestion times have been shown to alter transcriptomic signatures and induce stress responses that affect cell classification. Solving these challenges would help drive the future of tissue mapping and disease diagnostics through the field of single-cell sequencing. Therefore, new approaches and technologies are urgently needed to ensure the reliability and wide acceptance of single-cell tissue analysis methods. Single cell sequencing methods mainly focus on miRNA, but for single cell miRNA sequencing only very few protocols are available [15–19]. Enzyme-free tissue dissociation is a promising new approach for miRNA-Seq from archived frozen biopsy samples. This method uses mechanical force to dissociate tissue into single cells without the use of enzymes [20,21]. While miRNA molecules are often subjected to degradation processes during long-term storage, miRNA molecules, owing to their compact size, exhibit robust resistance to such degradation. This characteristic allows miRNA profiles to retain a reflection of the native biological landscape, presenting an invaluable opportunity to investigate the molecular states as they existed prior to the archival process. Another reason for emphasizing miRNA analysis lies in the regulatory role of miRNAs in gene expression. By examining miRNA profiles, one not only gains insights into the preservation of the biological state but also indirectly access information about gene expression profiles. miRNAs serve as mediators of post-transcriptional gene regulation, and variations in their abundance can have profound effects on the gene expression landscape. In essence, the choice to prioritize miRNA analysis in archived tissue samples is driven by the unique advantages of miRNA stability over time and their pivotal role as regulators of gene expression. This would be particularly important for the transfer of single-cell diagnostics to human samples in the clinical setting.

This technical brief details the methods and procedures employed for the investigation of human tissue samples preserved in RNALater at –80 °C, and processed for single-cell miRNA sequencing. The study aimed to assess a proof of concept workflow for an enzyme free tissue-to-single-cell conversion using a TissueGrinder and subsequent isolation of individual cells for miRNA-Seq analysis.

2. Material and methods

2.1. Human sample collection

Tissue samples from two human donors of the miRNAAtlas2 program and a 100 µm filter was used. Next, the TissueGrinder tube was centrifuged for 5 min at 300 g. The grinding gear was opened, washed with 3–5 ml PBS and centrifuged again for 1 min. The cell count and the amount of dead cells of the resulting single cell suspension were determined using a Countess (Invitrogen).

2.2. Single cell isolation

A live-dead staining was performed using Trypan Blue Stain 0.4 % (Invitrogen). Cell suspensions were placed on AdcellTM diagnostic slides (Thermo Fisher Scientific). Single living cells were isolated under the microscope in 1 µl PBS using a micromanipulator (Patchman NP2) with pump (CellTram, both Eppendorf) and placed into 2 µl of lysis buffer (0.2 % Triton X-100 (Sigma-Aldrich)) and 4 U recombinant RNase inhibitor (Clontech Takara). Samples were stored at –80 °C for up to six months.

2.4. Library preparation for miRNA sequencing

The preparation of the single cell miRNA sequencing libraries was performed as described in Hücke et al., 2021, Nature Comm. [15] for the SBN_C1 protocol with minor modifications. First, 5.8S rRNA masking and 3′ CL adapter ligation were performed. To prepare the Excess Adapter Removal mix, 5 µM RT primer (Biotectors), 0.2 U 1 µl-1 RNase I and 5 mM DTT (both Biozym) were mixed and incubated at 37 °C for 30 min, at 70 °C for 20 min and forever at 8 °C was performed. Then, 0.5 µl 5 µM-1 Lambda Exonuclease and 0.5 µl 50 U µl-1 5′ Deadenylylase (both NEB) were added to the mix per sample. 2 µl of the Excess Adapter Removal mix were pipetted to every sample and incubated for 15 min at 30 °C, for 15 min at 37 °C and forever at 8 °C. Ligation of the 5′ CL adapter and reverse transcription were performed as described in the original SBN CL protocol. For the first PCR amplification, 12.5 µl per sample of the following mix were prepared: 1 µl RP1 oligo (Biotectors), 1x Phusion HF Buffer (Thermo Fisher Scientific), 0.15 mM dNTPs (Roche), 1.3 U RNase I and 5 mM DTT (both Biozym) and incubated for 30 min at 37 °C, for 20 min at 70 °C and forever at 8 °C. Next, 1 U Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) per sample were added. Then, 13 µl of the PCR1 mix were pipetted to every sample (total volume 30 µl) and the following PCR program was used: 30 s at 98 °C, 13 cycles of 10 s at 98 °C, 30 s at 60 °C, 30 s at 72 °C, followed by a final elongation of 5 min at 72 °C and storage at 8 °C. For the second PCR amplification, 23.25 µl per sample of the PCR2 mix were prepared: 0.8 µl RP1 oligo (Biotectors), 1x Phusion HF Buffer (Thermo Fisher Scientific), 0.2 mM dNTPs (Roche), 2.35 U RNase I and 5 mM DTT (both Biozym) and incubated for 30 min at 37 °C, for 20 min at 70 °C and forever at 8 °C. Next, 0.5 U Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) per sample were added. Then, 23.5 µl PCR2 mix were pipetted in a fresh 0.2 ml PCR tube and 1 µl sample after PCR1 and 0.5 µl of an 100 µM individual index primer were added. For PCR2 the following program was used: 30 s at 98 °C, 13 cycles of 10 s at 98 °C, 30 s at 67 °C, 30 s at 72 °C, followed by a final elongation of 5 min at 72 °C and storage at 8 °C. Finally, Ampure XP Bead (Beckman Coulter) size selection was performed using bead to sample ratios of 1x and 1.6x.

The concentration of every miRNA library was determined using the Qubit High Sensitivity DNA kit (Life Technologies). If necessary, the samples were diluted to 1.8 ng µl−1 and the fragment length distribution was evaluated on a Bioanalyzer High Sensitivity dsDNA chip (Agilent Technologies).

2.5. Sequencing

The miRNA libraries were quantified using the KAPA Library Quantification Kit for Illumina (Roche). All libraries were pooled in equimolar concentration and sequenced on an Illumina MiSeq instrument. A final concentration of 18 pM library spiked with 5 % PhiX (Illumina) was sequenced (75 bp, single read) using a MiSeq Reagent Kit
deduplication was performed with UMI-tools (1.0.0). Data were quantified with mirquantify (1.0.1) from miRMaster (2.0) [23], and from GENCODE, miRBase, piRBase, and GtRNAdb. Unannotated regions classes were assigned using featureCounts (1.5.2) based on annotations.

2.6. Bioinformatic analysis

The bioinformatic analysis of the cell miRNA sequencing libraries were conducted following the methodology outlined in Hücke et al., 2021, published in Nature Communications [15]. Here, a concise summary of the described procedures is provided for clarity and reference. In the Bioinformatics analysis, Illumina BCL files were converted to FASTQ format using bcl2fastq (2.19.0.316). Reads with unique molecular identifiers (UMIs) had their UMIs incorporated into the header. After adapter and quality trimming with cutadapt (2.1042), reads were mapped to the human genome (GRCh38) using STAR (2.7.5b). RNA classes were assigned using featureCounts (1.5.2) based on annotations from GENCODE, miRBase, piRBase, and GtRNAdb. Unannotated regions were labeled "unmapped" Reads were hierarchically assigned to categories, with prioritization in cases of ambiguity. miRNA expression was quantified with mirquantify (1.0.1) from miRMaster (2.0) [23], and deduplication was performed with UMTools (1.0.0). Data were analyzed with and without subsampling using R and Seurat (4.4.0), and various visualizations and analysis were conducted for miRNA variability, clustering, and enrichment.

3. Results

In order to investigate the feasibility of converting tissues, initially stored in RNALater at –80 °C for two years, into a single-cell suspension a TissueGrinder was used. Subsequently, the isolated individual cells were processed for analysis in miRNA lysis buffer, paving the way for miRNA-Seq investigations. This workflow (Fig. 1) outlines a comprehensive procedure for obtaining high-quality miRNA-Seq data from RNALater-stored tissues. Initially, following rapid autopsy, tissue samples undergo immediate immersion in RNALater, ensuring the stabilization of RNA. Subsequently, these RNALater-preserved tissues are stored at –80 °C until required for processing. Upon thawing, tissues undergo effective dissociation into a single-cell suspension facilitated by the TissueGrinder. The TissueGrinder’s grinding unit exerts controlled mechanical forces, ensuring gentle yet effective disruption of tissue structures, thereby maximizing cell yield and preserving cellular viability. This method is a key component and contributes to the overall success of the proof of concept workflow, providing a technological advancement in single-cell dissociation for subsequent miRNA-Seq analysis from RNALater-stored tissues. The generated single-cell population is then picked from the suspension using a micromanipulator. Following this, single cells are lysed in a specialized miRNA lysis buffer, and the resultant lysates are stored at –80 °C until they undergo library preparation. Libraries are prepared using a dedicated miRNA-Seq kit, and the generated sequences are subjected to next-generation sequencing (NGS). The resulting miRNA-Seq data are subjected to thorough analysis, enabling the identification of differentially expressed miRNAs and an exploration of their biological significance. This integrated workflow stands as a robust and reproducible approach for single-cell miRNA-Seq analysis from RNALater-stored tissues.

3.1. Sample overview

Table 1 presents a comprehensive summary of the samples included in the study, outlining the experimental protocols for tissue digestion (TG Protocols), cell viability assessments, cell yield data, and the specific cells selected for subsequent miRNA sequencing analysis. The tissues exhibited reduced rigidity compared to fresh specimens, necessitating a cautious and gentle dissociation approach. For the liver and spleen, the method resulted in a uniform single-cell suspension, although the liver exhibited a reduced proportion of viable cells, indicating increased susceptibility to freezing. Conversely, the cortex presented a more challenging scenario, with only a limited number of viable individual cells observed under the microscope, potentially attributed to the higher fat content in brain tissues, thereby increasing the complexity of the dissociation process.

3.2. Quality control miRNA sequencing

Quality control in miRNA sequencing is a critical step that ensures the reliability and accuracy of the obtained miRNA data. This process involves the assessment of sequence data, the removal of low-quality reads, and the validation of miRNA profiles, ultimately guaranteeing the robustness of downstream analysis. Beyond general quality control measures, our approach incorporates a bioinformatical evaluation of sample quality. Prior to downstream analysis, we assessed the distribution of base quality scores, identified and discarded low-quality reads, and validated miRNA profiles. This stringent quality control framework not only enhances the reliability of miRNA expression data but also addresses potential biases introduced during library preparation and sequencing. By implementing these measures, we ensure the generation of high-quality data, thereby fortifying the foundation for subsequent bioinformatic analysis and contributing to the overall robustness of the study’s findings.

Fig. 2A presents the total count of miRNA Seq reads obtained per

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**Fig. 1.** To examine the feasibility of converting tissues, obtained from Saarbrücken University, preserved in RNALater at –80 °C, into a single-cell suspension utilizing a TissueGrinder, with subsequent isolation of individual cells into miRNA lysis buffer for miRNA-Seq analysis. (Image Source: Created with BioRender.com).
individual cell, which averages at approximately 600,000 reads per cell. Notably, with the exception of two outliers observed in the liver samples, the distribution of read counts across the cells exhibits a consistent and uniform pattern, aligning with our anticipated expectations. Fig. 2B depicts the initial bioinformatic assessment conducted subsequent to sequencing. During this quality control phase, some reads are excluded due to their inadequate data quality, indicating a substantial level of inaccuracy in the determination of the DNA sequence at the specific position (indicated in light grey, and designated as 'lost in QC'). An intriguing characteristic of miRNA libraries is the relatively elevated occurrence of 'empty' reads, in which only the adapters were sequenced, without any accompanying insert. Purification methods are ineffectual in eliminating these reads, given that their size differs by only 20 base pairs from those containing an insert (illustrated in dark grey as 'adapter dimers'). In our miRNA-Seq protocol, chemically modified adapters and a step to remove unligated 3′ adapter are used to reduce the amount of adapter dimers and miRNAs. This data is now ready for further analysis to identify differentially expressed miRNAs between cell types.

### 3.3. Mapping of reads and number of miRNA per cell

Mapping of reads and determination of the number of miRNA per cell is a fundamental aspect of miRNA sequencing analysis. This step involves aligning sequencing reads to a reference genome or miRNA database to identify and quantify miRNA species within individual cells. The results provide insights into the miRNA expression landscape, enabling the characterization of tissue-specific miRNA profiles.

Within this section, a comprehensive breakdown of the reads that successfully preprocessed, with a high percentage of reads mapped to the reference genome and a low percentage of adapter dimers and miRNAs. This data is now ready for further analysis to identify differentially expressed miRNAs between cell types.

### Table 1

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>RIN</th>
<th>Dissociation protocol</th>
<th>Filter [µm]</th>
<th>Cell Yield [cells/ml]</th>
<th>Viability [%]</th>
<th>Number of picked single cells [-]</th>
<th>Number of sequenced single cells [-]</th>
<th>Sample ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>3,9</td>
<td>Liver Modified</td>
<td>70</td>
<td>3,99E+05</td>
<td>30 %</td>
<td>10</td>
<td>20</td>
<td>Liver SC11-10,Liver</td>
</tr>
<tr>
<td>Spleen</td>
<td>2,4</td>
<td>Spleen modified</td>
<td>70</td>
<td>2,58E+05</td>
<td>93 %</td>
<td>10</td>
<td>10*</td>
<td>Spleen SC11-20,Spleen modified</td>
</tr>
<tr>
<td>Cortex</td>
<td>5</td>
<td>Spleen modified</td>
<td>100</td>
<td>6,45E+04</td>
<td>64 %</td>
<td>3</td>
<td>3</td>
<td>Cortex SC1-3,Spleen</td>
</tr>
</tbody>
</table>

Fig. 2. Summarizing the results of single-cell miRNA sequencing data preprocessing. A) The chart shows the number of sequenced reads for each tissue sample, B) Number of reads lost in quality control (QC) and mapped to the reference genome. The chart also shows the percentage of reads that were adapter dimers and miRNAs.

In Fig. 4A we conducted a UMAP analysis with the aim of simplifying the data’s complexity. The resulting two-dimensional plot reveals only the values that exhibit the greatest dissimilarity among individual cells.
within this dataset. The analysis distinctly illustrates that cells originating from the same tissue consistently cluster together, indicating a higher degree of similarity and shared characteristics compared to cells derived from distinct tissues. Within Fig. 4B–D, we provide an insight into the number of distinct miRNAs detected within individual cells, categorized by the three respective tissue types. For single liver and cortex cells, more different miRNAs could be detected compared to single spleen cells. Notably, these numerical findings align with the miRNA diversity observed in other single-cell studies, underlining the consistency and congruity of our results in comparison to broader research in the field.

4. Discussion

The findings presented in this study have substantial implications for the preservation and analysis of tissues for miRNA research. Our
investigation revealed that tissues stored in RNALater at −80°C for an extended period of approximately two years maintained the potential for extracting single-cell suspensions, demonstrating robust cell viability suitable for single-cell miRNA analysis with the enzyme-free TissueGrinder technique.

Single-cell miRNA-seq necessitates the effective integration of two distinct techniques: the isolation of specific individual cells from cultures, tissues, or dissociated cell suspensions, and subsequently, the conversion of the limited cellular RNA into cDNA, followed by the high-throughput sequencing of cDNA libraries [24]. In deviation from earlier published research, this study provides the initial evidence supporting enzyme-free tissue dissociation followed by miRNA sequencing. Enzymes are commonly employed for tissue dissociation, requiring incubation at 37°C for variable durations depending on the tissue type. This temperature activates the cellular transcriptional machinery, potentially leading to alterations in gene expression in response to the dissociation process and other environmental stresses [25,26]. As opposed to enzyme-based approaches, we hypothesize that rapid and enzyme-free dissociation enables improved control over pre-analytic variables, facilitates standardization, and minimizes transcriptional drift [27,28].

Nevertheless, a comprehensive exploration of these aspects awaits further investigation. In this study, we successfully demonstrated the feasibility in principle, utilizing various archived tissue samples.

In case of tissue samples, preparation and isolation of single cells are additional very difficult steps in single-cell analysis [29]. The ability to effectively preserve tissue samples for this duration without compromising cell viability and miRNA integrity is a noteworthy achievement. This feature extends the window of opportunity for researchers to access archived tissue specimens and perform in-depth miRNA-Seq analysis. The detection of specific mutations or mutation spectra and/or perturbed gene expression profiles is crucial for the selection of modern drugs for personalised therapy [30]. This has significant implications for studies requiring long-term sample storage, retrospective analysis, and the utilization of biobanks with archived samples.

Importantly, the miRNA profiles obtained from these preserved tissues displayed a level of comparability with miRNA data from other cell cultures and research results performed with fresh cells [15]. This consistency underscores the reliability and validity of the preservation and isolation methods employed in our study. It indicates that the miRNA profiles derived from the preserved tissues are representative of the biological information they contain and are not significantly altered during the storage period.

5. Limitations

The technical brief faced several limitations that warrant acknowledgment. Firstly, the availability of tissue samples from healthy body donors through a rapid autopsy program inherently restricted the size and diversity of our sample pool. Additionally, the time frame between sample removal, processing and RNA stabilization remains crucial for preserving RNA integrity. Despite efforts to minimize time frames, the logistical challenges entail that not all preserved samples present the necessary quality for downstream analysis. In order to draw biologically meaningful conclusions, a significantly larger number of samples or cells from different healthy donors, comprising different age groups and genders, would have been required. Since we directly used a single cell suspension from disaggregated tissue for miRNA analysis without upstream fluorescence-activated cell sorting (FACS) to select different cell types, we could not analyze a sufficient number of single cells per cell type, which limited the statistical interpretability of our results. Addressing these limitations in future studies could provide a more comprehensive understanding of miRNA expression patterns in different cell types derived from healthy donor tissues, thereby advancing our knowledge of physiological processes of cellular behavior and cellular heterogeneity within organs.

6. Conclusion

In conclusion, our study demonstrates the feasibility of preserving tissues in RNALater at −80°C for an extended duration of approximately two years while maintaining the integrity of miRNA. This preservation method, coupled with the TissueGrinder technique, offers an effective means of converting tissues into single-cell suspensions for miRNA-Seq analysis. Moreover, our results emphasize the comparability of miRNA profiles obtained from these preserved tissues with those derived from other sources, confirming the utility and robustness of this preservation and isolation approach in advancing miRNA-related research. This approach holds the potential to benefit a wide range of scientific investigations and the long-term storage of invaluable tissue specimens. Single cell miRNA-seq could be used to identify specific cell types in which miRNAs are highly expressed. This information could be used to develop new biomarkers for diseases that are specific to certain cell types. Additionally, single cell RNA-seq could be used to study the effects of drugs and other treatments on miRNA expression in individual cells. This information could be used to develop new and more personalized treatments for diseases.

CRediT authorship contribution statement

Stefan Scheuermann: Conceptualization, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. Sarah Hücke: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. Annika Engel: Data curation, Formal analysis, Software, Visualization, Writing – original draft. Nicole Ludwig: Data curation, Formal analysis, Software, Visualization, Writing – original draft. Philipp Lebhardt: Conceptualization, Writing – original draft. Jens Langejürgen: Project administration, Resources, Writing – original draft. Stefan Kirsch: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stefan Scheuermann reports a relationship with Fast Forward Discoveries GmbH that includes: employment and equity or stocks. Jens Langejürgen reports a relationship with Fast Forward Discoveries GmbH that includes: equity or stocks. Stefan Scheuermann has patent #WO2019145029A1 pending to Fast Forward Discoveries GmbH. S.S. and J.L. are co-founders of the company Fast Forward Discoveries GmbH, which commercializes the TissueGrinder technology. The other authors declare no competing interests. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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