Single-cell RNA sequencing of intramedullary canal tissue to improve methods for studying fracture repair biology

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First draft submitted: 8 January 2021; Accepted for publication: 23 June 2021; Published online: 10 August 2021

ABSTRACT
The ability to study the bone microenvironment of failed fracture healing may lead to biomarkers for fracture nonunion. Herein the authors describe a technique for isolating individual cells suitable for single-cell RNA sequencing analyses from intramedullary canal tissue collected by reaming during surgery. The purpose was to detail challenges and solutions inherent to the collection and processing of intramedullary canal tissue samples. The authors then examined single-cell RNA sequencing data from fresh and reanimated samples to demonstrate the feasibility of this approach for prospective studies.

METHOD SUMMARY
Intramedullary canal tissue is challenging to study directly because of its inaccessibility and heterogeneous composition. In addition, single-cell RNA sequencing requires high sample purity and cell viability. The authors determined that the critical step required for producing usable samples for single-cell RNA sequencing from intramedullary canal tissue was collagenase digestion, followed by centrifugation with density gradient medium (Ficoll).

KEYWORDS:
cell isolation • cryopreservation • fracture • nonunion • single-cell RNA sequencing

Nonunion of lower extremity fractures is a debilitating orthopedic condition. Patients experience prolonged pain, physical disability and decreased quality of life of the same magnitude as congestive heart failure [1]. Implicit to lower limb nonunions are long hospital stays and long-term unemployment, outcomes that are relevant to the public economy [2,3]. A practical definition of nonunion is when a fracture, in the opinion of the treating physician, has no possibility of healing without further intervention [4]. The overall risk estimate for nonunion in adults is 1.9% of all fractures, or 500,000 cases annually [5,6]. Clinical risk factors include injury characteristics and host factors, including age [7], fracture site (e.g., tibia/fibula [5.4%], femur/pelvis [1.3%]) [6], type of injury, open versus closed fracture [8], smoking status [9] and patient comorbidities [8]. However, the cellular biology of impaired fracture healing remains only marginally understood.

Once identified, nonunion is largely a surgical disease. Frequently, the procedure for nonunion repair involves accessing the intramedullary canal and reaming for implant removal, exchange nailing or obtaining autogenous bone graft. The primary goal of the present study was to determine the feasibility of collecting human intramedullary canal tissue (ICT) for single-cell RNA sequencing (scRNA-seq), looking toward improved understanding of cellular mechanisms in fracture repair or biomarkers predictive of failed healing. To demonstrate the applicability of this technique for downstream analyses, the authors first defined a suitable cell isolation protocol, determined cell viability and then performed scRNA-seq analyses. This is a time-intensive endeavor that involves coordination between multiple entities (surgeons, laboratory personnel and scRNA-seq core facility). Therefore, a secondary goal was to investigate whether freezing and subsequent reanimation of samples would allow for scRNA-seq. Taken together, these two goals have allowed the authors to collect, store and process ICT samples for future research. The authors have successfully completed seven additional sample acquisitions and analyses using the protocol described in this brief report.

Human samples
Deidentified samples of ICT were obtained from three patients. The three patient samples consisted of the following: femur nonunion sample was ICT from a femoral nonunion undergoing exchange intramedullary nailing for treatment of the nonunion; femur control
Intramedullary canal tissue (ICT) samples were fixed for 24 h in neutral buffered formalin (10%) and serially dehydrated in 5, 15 and 30% sucrose–phosphate-buffered saline for 24 h. Samples were embedded in optimal cutting temperature medium, frozen on dry ice and ultimately sectioned via cryostat at 10-μm thickness. After sectioning, slides were stained with hematoxylin and eosin. Images were obtained using a Leica DMS5000 B microscope, FLUOTAR objectives, DFC310 FX camera and LAS X software. Cells (blue) are surrounded by both soft tissue (pink) and hard tissue (red), indicating that the ICT obtained from reaming is a complex matrix. Black arrows indicate dense collections of cells.

To troubleshoot and validate the cell isolation procedure, the products were pelleted and then resuspended and incubated in Hoechst 33342 at 8 mM in Dulbecco’s modified Eagle medium (DMEM) for 5 min. Cells were thereafter washed once, resuspended in DMEM and examined by microscopy. Images were obtained using a Leica DMS5000 B microscope, FLUOTAR objectives, DFC310 FX camera and LAS X software. (A) Representative image of Hoechst-labeled live cells from intramedullary canal tissue sample filtrate without enzymatic digestion, revealing mostly erythrocytes. (B) After enzymatic digestion, demonstrating the dense mineral debris that contaminates the live cell pellet. (C) After utilization of both enzymatic digestion and subsequent Ficoll density gradient medium separation. The latter combination successfully and consistently partitioned the desired fraction of viable cells away from the mineral debris. Scale bars = 20 μm.

sample was from a normal, previously unoperated femur from which autogenous bone graft was obtained for a contralateral femur nonunion site; and acute tibia fracture sample was collected at the time of intramedullary nail (IMN) fixation. Reaming was performed using flexible reamers (Zimmer Biomet, IN, USA). For the nonunion sample, the previously placed IMN was removed, and the canal
was then reamed in preparation for receipt of a new, larger IMN. For the control sample, a standard reamer was passed prior to bone graft harvest with the Synthes reamer-irrigator-aspirator (DePuy Synthes, PA, USA), with the initial reamings from the standard reamer constituting the ICT sample. Finally, the acute tibia fracture underwent reaming for standard IMN placement for fracture treatment. In all cases, ICT was collected from the reamings, immediately placed on ice in 50-ml conical tubes and transferred to the laboratory for tissue processing. All specimens were from samples routinely collected and discarded at the time of surgery, and all were deidentified for this investigation.

**Sample preparation**

The necessary supplies and reagents are shown in the reagents and materials in the protocol template. The cell isolation protocol from the ICT went through a series of trial and error revisions to obtain suitable live cell yield and minimize contaminating mineral debris. The authors’ goal was to obtain a protocol that would allow cell isolation from ICT and pass scRNA-seq quality control (QC). The primary error in the design of the initial protocol was due to the assumption that cells would be organized in an easily dispersible matrix, as one would encounter with a typical bone marrow aspiration sample [10]. However, after fixing, staining and observing the ICT samples via microscopy (Figure 1), it became evident that the majority of cells were unable to be extracted by physical manipulation alone. The second major obstacle was partitioning viable cells away from contaminating mineral debris, which is voluminous and an inherent byproduct of the reaming procedure. Ultimately, a collagenase digestion followed by Ficoll density gradient separation liberated single cells and allowed for the separation of mineral debris from viable cells. Figure 2A & B displays the cellular endpoints from early failed isolations, and Figure 2C reveals the product of the final successful protocol. The final protocol, as described, was used to isolate cells from ICT for all three patient samples. Each of the three samples (acute tibia fracture, femur control and femur nonunion) was divided into samples that were freshly analyzed and samples frozen at -80°C for greater than 3 weeks. The frozen samples were then thawed, reanimated and analyzed again. Thus, the same isolation protocol was used for each of the three samples (both fresh and frozen).

The number of cells expected for the 10x Chromium system (10x Genomics, CA, USA) varies from 100 to 10,000, depending on cell availability and the need for single-cell gene expression analysis. The Chromium system requires 60 μl of a clean single-cell suspension at 700–1200 cells/μl. The definition of clean single-cell suspension is as follows: approximately 90% viability; zero to minimal cell aggregation, particularly doublets and triplets; minimal cell debris; and final cell suspension washed at least three times. Often, when obtaining a sample from a reaming procedure, the initial pellet is very large but consists almost entirely of mineral debris. In the later steps, the pellet is often very small and barely visible. Thus, pellet size (or absence thereof) is not a criterion for successful isolation. If the cell pellet is not visible, the final cell count by hemocytometer is used to ensure adequate cell number. In the fresh state, the number of cells that passed QC was 3156, 4515 and 7770 for the acute tibia fracture, femur control and femur nonunion samples, respectively. Following reanimation, the number of cells that passed QC was 6201, 7204 and 13,006 for the acute tibia fracture, femur control and femur nonunion samples, respectively.

**scRNA-seq & bioinformatics**

To coordinate the services of a multidepartment core facility with the time-intensive sample preparation and challenges inherent to surgical scheduling, it is ideal to be able to freeze samples after isolation for later analysis. To investigate whether the freeze/thaw process compromised cell viability or impaired downstream analyses, the authors compared samples analyzed on the day of collection (never frozen) with the same sample stored for greater than 3 weeks at -80°C and subsequently thawed per the recommendations of 10x Genomics [11]. Cell viability was determined manually by the core facility via trypan blue and hemocytometer prior to downstream analysis. The fresh versus reanimated cell viability was 84 versus 78% for femur nonunion, 91 versus 90% for the acute fracture and 92 versus 61% for the control sample. Of note, because of the lower percentage of viable cells after freezing the control sample, the core utilized a dead cell removal kit (Miltenyi Biotec, Auburn, CA, USA), which increased viability to 93% in the control sample used for scRNA-seq. Each sample/cell suspension was processed according to the manufacturer’s instructions, as previously described [12,13]. Briefly, each sample was loaded onto a Chromium chip (10x Genomics). The 10x V3 single-cell reagent kit (10x Genomics) was used to generate single-cell gel beads for cDNA synthesis. An Agilent 2100 Bioanalyzer (Agilent, CA, USA) was used to assess the subsequent Illumina libraries, which were then sequenced via an Illumina NovaSeq 6000 (Illumina, CA, USA).

The data were analyzed with Cell Ranger 3.0.2 [14], with slight modifications from previous reports [12,13]. Briefly, FASTQ files were aligned to the human reference genome GRCh38. The aligned reads were traced back to individual cells, and gene expression was quantified based on the number of unique molecular indexes detected in each cell. R package Seurat 3.1.0 [15] with RStudio 1.1.453 and R 3.5.1 was used to further analyze the filtered gene–cell barcode matrices. Exclusion criteria were genes detected in less than five cells, cells with less than 200 genes, cells with extremely high or low numbers of detected genes/unique molecular identifiers and cells with high percentages of mitochondrial reads. The function ‘is Outlier’ from R package scater [16] was used in this process of data clean-up. Following exclusion as described, the resultant data were normalized with the NormalizeData function in Seurat. FindIntegrationAnchors and IntegrateData from Seurat 3.1.0 integrated each pair of fresh and reanimated samples. The integrated data were scaled and principal component analysis was performed. FindNeighbors and FindClusters were used to identify clusters. FindConservedMarkers was subsequently used to identify canonical cell type marker genes. The cell clusters were visualized using the Uniform Manifold Approximation and Projection plots. R package ggplot2 [17] was used to plot the percentage of cells in each cluster.
Figure 3. Comparison of single-cell RNA sequencing cell clusters in fresh versus reanimated samples. Uniform manifold approximation and projection comparisons of scRNA-seq analyses of the three pairs of fresh and reanimated samples. Each dot represents a cell, and each number/color represents clusters of similar cell types determined by gene expression profile, referenced to a standard data set. The figures demonstrate similar clustering in the samples when analyzed fresh or after reanimation. (A) Control femur sample, fresh; (B) control femur sample, reanimated; (C) nonunion femur sample, fresh; (D) nonunion femur sample, reanimated; (E) acute tibia sample, fresh; (F) acute tibia sample, reanimated.
Figure 4. Comparison of cell percentages in each cluster in fresh versus reanimated samples. The values along the x- and y-axes are the percentages of either fresh (x-axis) or reanimated (y-axis) samples that a particular cluster represents. A cluster that has an identical percentage of cells (comparing frozen and reanimated samples) would be located along the diagonal hashed line. A cluster with a large deviation of percentages between the two samples would appear as farther away from the diagonal line. Most of the dots are distributed close to the line, suggesting that freezing had minimal effect on the proportion of cells from a cluster in the overall population. (A) Control femur sample, (B) nonunion femur sample and (C) acute tibia sample.

Figure 3 compares the Uniform Manifold Approximation and Projection plots for clusters between the fresh and reanimated samples, demonstrating relative stability of the clusters. Figure 4 shows that the percentage of cells from each cluster in the overall cell population was minimally affected by reanimation and close to the line of identity. Figure 5 uses violin plots to illustrate frequency distributions for the number of genes expressed in each cell. The black (fresh) and gray (reanimated) plots illustrate the distribution within each condition. The main finding of these data was that reanimation passed scRNA-seq QC and did not prevent subsequent analyses.

Improving our understanding of failed fracture healing will have a profound effect on the diagnosis and treatment of nonunion. One potential avenue of inquiry is studying scRNA-seq of ICT. In this article, the authors have described a novel, successful and repeatable operating room-to-benchtop process for the isolation of single cells from ICT collected at the time of reaming during nonunion repair.
Figure 5. Violin plots comparing the number of genes per cell in fresh versus reanimated samples. The black (fresh) and gray (reanimated) plots represent the proportion of cells that express a given number of genes (y-axis). These figures show minimal change when comparing the fresh samples with their reanimated counterparts. (A) Control femur sample, (B) nonunion femur sample and (C) acute tibia sample.

and acute fracture treatment. Through a series of adjustments, the authors successfully isolated single cells of adequate quality and quantity for scRNA-seq analyses. Most importantly, the authors showed that cryogenic storage of samples is possible, thereby facilitating the feasibility of this translational research technique. To the authors’ knowledge, there are no published protocols describing the isolation of cells from ICT obtained by reaming that are suitable for scRNA-seq analyses. The authors acknowledge that this protocol may not yet be optimal and welcome further investigation. Additionally, there remain aspects of the comparison between fresh and reanimated samples that are underdeveloped. However, this technique article demonstrates the ability to isolate cells from ICT suitable for scRNA-seq. The authors also acknowledge that reanimated samples may not fully reflect fresh samples; however, the differences appear minimal. Pragmatism in doing these types of experiments is a relevant concern. Although some scientific questions using this protocol may require fresh samples, others may be answerable with frozen samples. The authors’ protocol shows that cells passing scRNA-seq QC can be obtained from both types of samples.

Future perspective
This communication provides a protocol for isolating cells from ICT suitable for scRNA-seq analyses. Using this protocol, investigation of cell types in each cluster or gene expression differences between nonunions and controls may yield insight into, or biomarkers for,
failed fracture healing. Ongoing research in our laboratory using this methodology is examining differences between multiple control and nonunion patients.

Executive summary

- Analysis of unique cellular characteristics of intramedullary canal tissue from fracture nonunion patients may provide critical insight into the etiology of, or biomarkers for, failed fracture healing.
- The authors report the feasibility of collecting, processing and analyzing intramedullary canal tissue specimens using single-cell RNA sequencing in both fresh and reanimated conditions.

Author contributions

The study was conceived by RM Natoli, TO McKinley, Y Liu and SM Moe. Logistics were provided by KM Brown and NX Chen. Experiments were performed by JM Dominguez. Bioinformatics analysis was generated by H Gao. The manuscript was written by JM Dominguez, SM Moe and RM Natoli. All authors participated in data interpretation and revising the manuscript and approved the final version.

Disclaimer

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Financial & competing interests disclosure

This work was supported by grants from the Indiana Center for Musculoskeletal Health (multi-investigator pilot P30 award number AR072581); Indiana Clinical and Translational Sciences Institute (award number UL1TR002529); NIH, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award; Indiana Center for Musculoskeletal Health Collaborative Pilot Project Grant; and Indiana University Health Values Fund Grant (VFR-478). SM Moe is supported by P30AR072581, UL1TR00259 and R01DK110871. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. Samples obtained were discarded products of surgery and did not contain human subject identifiers.

Data sharing statement

RNA sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (accession number PRJNA540413).

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