

# Systematic Study of Single-Cell Isolation from Musculoskeletal Tissues for Single-Cell Sequencing

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## Research article

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# Abstract

**Background:** The single-cell platform provided revolutionary way to study cellular biology. Technologically, a sophistic protocol of isolating qualified single cells would be key to deliver to single-cell platform, which requires high cell viability, high cell yield and low content of cell aggregates or doublets. For musculoskeletal tissues, like bone, cartilage, nucleus pulposus, tendons, etc. as well as their pathological state, which are tense and dense, it's full of challenge to efficiently and rapidly prepare qualified single-cell suspension. Conventionally, enzymatic dissociation methods were wildly used but lack of quality control. In the present study, we designed specific enzymatic treatment protocols for several human pathological musculoskeletal tissues, including degenerated nucleus pulposus, ossifying posterior longitudinal ligament and knee articular cartilage with osteoarthritis, aiming to rapidly and efficiently harvest qualified single-cell suspensions to meet the requirements of single-cell RNA-sequencing (scRNA-seq).

**Results:** The single-cell suspensions from human degenerated nucleus pulposus and ossifying posterior longitudinal ligaments were both qualified after systematic quality control. Bioanalyzer trace showed expected cDNA size distribution of the scRNA-seq library. A clear separation of cellular barcodes from background partitions were verified by the barcode-rank plot after sequencing. However, we failed to obtain eligible samples from articular cartilage due to low cell viability and excessive cell aggregates and doublets.

**Conclusions:** In conclusion, we provided rapid and efficient single-cell isolation protocols for human degenerated nucleus pulposus and ossifying posterior longitudinal ligament, which could be applied for scRNA-seq. More efforts will be made on improving the protocols for human articular cartilage.

## Background

Since establishment of the scientific discipline of cellular biology and development of the cell theory in 1839 [1], the researchers' endeavor to enrich the field has never been halted. The milestone theory states that the cell is the basic unit of structure and organization of living organisms. In any multicellular organism, cell heterogeneity is one of the most prominent characteristics, which is critical to its peculiar function and fate. Similarly, living tissues are composed of diversiform cell types and each cell type is generally incorporated with genetically heterogeneous cell-subpopulations [2]. Due to this complexity, the conventional analyses of cellular biology using bulk of tissues or cells are apparently limited. The emerging field of single-cell techniques provides promising tools to characterize the cellular biology at the resolution of individual cells [3, 4].

Single-cell genomic technologies, coincided with transformative new methods to profile genetic, epigenetic, spatial, proteomic and lineage information, have revolutionized the research methodology for exploring biological systems [5]. Technologically, the workflow mainly includes four parts: tissue procurement and single-cell isolation, Single-Cell Sequencing library construction and quality control,

library sequencing, and data analysis. As rapid development and maturation of single-cell platform, high quality input of single-cell suspension has become the prerequisite factor for ideal output datasets [6]. For nonadherent cells like peripheral blood mononuclear cells, it's much easier to gain single-cell suspension. While for adherent and even solid tissues, it's a challenge to dissociate cells from extracellular matrix without injuring cell viability. Especially for musculoskeletal system, including bones, muscles, joints, cartilage, ligaments, tendons and bursae, tissues of which are dense and tense.

Commonly, protocols for tissue dissociation mainly include mechanical dissection, enzymatic breakdown and combinatorial methods. In any event, little damage and high efficiency are the baseline for viable single-cell isolation. Although, Magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) could be used to discard the dead cells to meet the requirements of single-cell platform, the resulting lost cells would compromise heterogeneity. Therefore, high cell viability should be defended during sample preparation. Traditionally, various enzymatic digestion protocols were employed for cell isolation from bone, cartilage, nucleus pulposus, and tendons, but generally lacked the quality control. In 2019, Ninib Baryawno et al. reported the Single-Cell RNA Sequencing (scRNA-seq) results of mice bone and bone marrow [7]. The enzymatic solution used in their study was 1 mg/mL STEMxyme1 (Worthington, Ref# LS004106) and 1 mg/mL Dispase II (ThermoFisher Scientific, Ref# 17105041) supplemented in Media 199 with 2% FBS (shown as the S-D solution in this paper). Taking advantage of the digestion medium, they rapidly obtained a highly viable and multifarious single-cell mixture with mesenchymal stem/stromal cells, osteo-lineage cells, chondrocytes, fibroblasts, endothelial cells, and pericytes, suggesting a broad-spectrum sensitivity and high efficiency to various tissues but moderate effect to cell viability.

Combined with the S-D solution and conventional methods, we designed specific enzymatic treatment protocols for single-cell isolation from human degenerated nucleus pulposus (NP), ossifying posterior longitudinal ligaments and knee articular cartilage with osteoarthritis (OA). Results showed that the single-cell suspensions from degenerated NP and ossifying posterior longitudinal ligament were qualified for single-cell platform. However, the single-cell suspensions from articular cartilage were unqualified due to excessive cell aggregates and doublets, suggesting insufficient digestion efficiency. To increase the efficiency, we prolonged the digestion duration, resulting in severely damaged cell viability. In conclusion, the single-cell isolation protocols for degenerated NP and ossifying posterior longitudinal ligament were validated for single-cell platform. However, the exploration for articular cartilage was failed, which needs more efforts in the future.

## Results

### 1. Validation of the single-cell isolation protocol for human degenerated NP

Human degenerated NP tissues (Figure 1B) were harvested during surgery from patients suffering degenerative disc disease (Figure 1A). After 3-5 cycles of digestion, the NP fragments were markedly diminished and only few white floccules were remnant (Figure 1C). The suspension became viscous,

which was probably resulted from increased concentration of proteoglycans disaggregated from extracellular matrix. After collection, filtration and wash, we harvested sufficient cells for following experiments ( $(1.20 \pm 0.15) \times 10^6$  cells counted automatically and  $(1.15 \pm 0.06) \times 10^6$  cells counted manually) (Figure 1D). According to the cell diameter distribution, cells with diameter  $\geq 20 \mu\text{m}$  occupied  $(6.6 \pm 0.6) \%$  (Figure 1E). Live/dead assays showed  $(91.7 \pm 3.2) \%$  of cells were Calcein<sup>+</sup> and  $(7.3 \pm 1.2) \%$  were PI<sup>+</sup>. There were few observed cell aggregates or doublets (Figure 1F), which corresponded with the cell diameter distribution. One of the qualified samples was sent to scRNA-seq library construction and sequencing. Quality of the library was validated by Agilent Bioanalyzer 2100 (Figure 1G). After sequencing, the barcode rank plot exhibited a steep slope (Figure 1H), suggesting a clear separation of cellular barcodes from background partitions.

## 2. Validation of the single-cell isolation protocol for human ossifying posterior longitudinal ligaments

Collagenase type  $\alpha$ , frequently used in digestion of bone and tendons, was combined with the S-D solution in the present study. Ossifying posterior longitudinal ligaments were obtained from patients with ossification of posterior longitudinal ligament (OPLL) during surgery (Figure 2A, 2B). After 3-5 cycles of digestion, only bone fragments and few soft tissues were left (Figure 2C, 2D). Total cell number counted automatically was  $(4.71 \pm 0.81) \times 10^5$  and manually was  $(3.70 \pm 0.39) \times 10^5$  (Figure 2E). The remnant bone fragments and modest cell yield were probably on account of confined enzymatic sensitivity to bone. However, longer duration or more intense enzymatic treatment would probably affect cell viability. Cells with diameter  $\geq 20 \mu\text{m}$  occupied  $(6.3 \pm 1) \%$  of total cells according to the cell diameter distribution (Figure 2F) and there were no observed cell aggregates or doublets (Figure 2G). High cell viability was verified by the live/dead assays, with  $(91.1 \pm 8.4) \%$  of cells Calcein<sup>+</sup> and  $(8.9 \pm 8.4) \%$  of cells PI<sup>+</sup> (Figure 2G). We then carried out scRNA-seq library construction and sequencing for one of the qualified samples. Bioanalyzer trace was shown to validate the library quality (Figure 2H). And barcode rank plot suggested a clear separation of cellular barcodes from background partitions (Figure 2I).

## 3. Failed single-cell preparation from human knee articular cartilage with OA

Collagenase type II was wildly used in dissociation articular cartilage, which was added with the S-D solution in this study. After digestion, filtration and wash, lots of cell aggregates were observed under microscope and only  $(83.0 \pm 3.8) \%$  of cells were viable (Figure 3C). Cells with diameter  $\geq 20 \mu\text{m}$  occupied  $(12.6 \pm 3.1) \%$  (Figure 3B). Fortunately, most of the cell aggregates were viable (Figure 3C), suggesting an insufficient digestive efficiency. According to Quanbo Ji's research, we prolonged the enzymatic treatment to increase the efficiency. The cell yield was significantly increased compared with the rapid treatment group (Figure 4A). However, the ratio of cells with diameter  $\geq 20 \mu\text{m}$  was unexpectedly increased to  $(22.6 \pm 2.2) \%$  (Figure 4B). Live/dead assays showed  $(65.2 \pm 10.6) \%$  of cells were dead (Figure 4C), including most of the cell aggregates.

# Discussion

Development of scRNA-seq technique contributes a big step for cellular biology. Preparation of qualified single-cell suspension is a precondition for the platform. It's full of challenge to offer eligible samples from dense and tense musculoskeletal tissues, like bone, cartilage, nucleus pulposus, tendons, etc. In the present study, we aimed to provide standard and specific protocols for preparing qualified single-cell suspensions from human degenerated nucleus pulposus, ossifying posterior longitudinal ligaments and knee articular cartilage with OA. As a result, the protocols for the former two types of tissues could be applied to output qualified samples for following scRNA-seq. However, dissociated cell suspensions from knee articular cartilage were unqualified due to either excessive aggregates and doubles or severely damaged cell viability, which needed more efforts in the future.

Nucleus pulposus, the inner part of intervertebral disc, performs critical biomechanical functions [8–10]. Accumulated evidences supported that the nucleus pulposus degenerated at early stage of degenerative disc disease [11–13], which is a common cause of disability and results in a huge socioeconomic impact world widely [14]. The etiology as well as pathology is complex and multifactorial [9, 15]. Although the underlying pathology has been approached from a variety of ways [9], a systematically study to get a panorama is still missing. The single-cell platform provided a revolutionary way to solve this problem, but there was no related report till now. Therefore, to harvest qualified single-cell suspension would be the first step. In previous studies, NP was usually dissociated via various enzymatic methods and the treatment duration was from 30 min to overnight, which was summarized detailly in Juliana's paper [16]. As is well known, long time and intense enzymatic treatment would increase cell yield while damage cell viability, vice versa. And the lack of systematic quality control made it hard to be directly used in single-cell isolation.

Pronase, a mixture of several nonspecific endo- and exoproteases, has been wildly used in isolating cells from NP [17]. It was reported that Pronase treatment might shorten the required digestion time [16]. Based on these researches, we combined Pronase with the S-D solution for single-cell isolation from NP in this study and obtained highly qualified single-cell suspensions possessing abundant cell yield, high cell viability, and few cell aggregates or doublets. In conclusion, the enzymatic treatment protocol for human NP was validated for single-cell platform.

The posterior longitudinal ligament runs entire length of the spine. Ossification of the posterior longitudinal ligament (OPLL), defined as an ectopic ossification (calcification), would lead to paralysis of extremities and disturbances of motility [18]. Up to now, the osteo-lineage derivation, cell atlas transformation and molecular regulation have never been elucidated, which retarded the development of etiological treatment [18–20]. Single-cell platform, taking advantage of horizontal cell atlas and vertical trajectory analysis, would broaden the horizons in this field. Normal posterior longitudinal ligament is fibrous connective tissue. However, the ossifying posterior longitudinal ligament is a mixture of ligament, bone and probably cartilage as a result of endochondral ossification process [21, 22]. It's full of challenge to ascertain one digestion medium to dissociate mixed tissues. Collagenase type  $\text{X}$ , frequently used in digestion of bone and tendons, was combined with the S-D solution in the present study. Moderate cell yield, high cell viability and low content of cell aggregates or doublets were realized using the present

protocol. Although scRNA-seq was conducted, the cell heterogeneity should be inspected carefully by data analysis. In particular, the quantity and variety of bone derived cells should be noted in consideration of lots of bone-like tissue remnants. More experiments will be carried out to raise the cell yield but not compromise on cell viability.

Articular cartilage is specialized connective tissue of diarthrodial joints and functions as a load-bearing, low-friction, and wear-resistant surface to facilitate joint movement [23]. As it is lack of self-regenerative ability, articular cartilage is one of the most common degenerative tissues. Osteoarthritis (OA) is the primary joint disorder and a major cause of disability, affecting 303 million people globally in 2017 [24, 25]. The etiology, pathology and regenerative therapy have been widely studied [26–29]. Also utilizing the single-cell platform, there were two nice reports that systematically uncovered the cell atlas and transcriptional regulation mechanisms of OA [30, 31]. As for other related studies, several reports showed the single-cell profiling results of developing limbs, including growth plate cartilage as well as articular cartilage [32–35]. The single-cell isolation methods used in the above studies were summarized in Table 1. In Quanbo Ji's research, the articular cartilage was obtained from 10 patients but only 1600 cells were sequenced, suggesting a too moderate digestive efficiency. In Fiorella Carla Grandi's study, the primary chondrocytes were delivered to culture and treatment in vitro before scRNA-seq, the quality control of isolated primary single cells was not reported. And in other researches, samples were obtained from mice but not human.

Table 1

Summary of Single-cell Isolation Protocols for Cartilage in Reported Studies Using scRNA-seq Technique

Reference	Tissue	Species	Treatment	Number of profiling cells
Quanbo Ji, et al. [30]	Cartilage from 10 knee OA patients	Human	0.25% Trypsin-EDTA for 30 min and then 0.2% type II collagenase (Sigma-Aldrich) for 4 h.	1600
Fiorella Carla Grandi, et al. [31]	OA samples from patients undergoing total joint replacement	Human	Collagenase II and IV (2.5 mg/ml each; Worthington Biochem) overnight.	Not reported
Junxiang Li, et al. [32]	The distal cartilage structure at postnatal day 7.	Mouse	0.2% collagenase II for 2 hours	217
Vikram Sunkara, et al. [33]	Femur of 12 weeks old mice	Mouse	10 mg/ml Collagenase II (Nordmark) supplemented for 4 h	7133
Koji Mizuhashi, et al. [34]	Femur growth plate cells at postnatal day 2.	Mouse	Liberase TM (Sigma/Roche) for unclear time	18,000
Natalie H Kelly, et al. [35]	Hindlimb bud of embryonic mice	Mouse	Collagenase Type II (Worthington-biochem, Lakewood, NJ) and pronase (EMD Millipore, Billerica, MA) in 15 min increments with agitation for up to 1 h	9466

Collagenase type II was widely used in dissociation articular cartilage, which was added with the S-D solution in this study. Using the rapid protocol, excessive live cell aggregates suggested insufficient enzymatic efficiency but longer duration of treatment significantly damaged cell viability with plenty of dead cell aggregates. Different from insufficient digestion, the dead cells could be aggregated into various sizes by released DNA. DNase could be used to degrade DNA and reduce dead cell aggregation, and FACS or MACS could be used to remove the dead cells. However, the potential loss of cell heterogeneity might mislead the data analysis. So longer enzymatic duration was not a better choice.

For hard dissociated tissues, single-nucleus RNA-sequencing (snRNA-seq) is an appealing alternative choice. In this method, a mild and quick nuclear dissociation protocol is used to isolate and sequence RNA within the nucleus, which minimizes technical issues that can arise from common dissociation protocols [36–37]. More detailed explorations will be performed in the future.

## Conclusions

The single-cell platform provided revolutionary way to study cellular biology at the resolution of individual cells. A thorough protocol for preparing qualified single-cell suspensions is prerequisite. In the present

study, we validated the enzymatic treatment protocols for preparing qualified single-cell suspensions from human NP and OPLL samples, which were rapid, efficient and easy to operate. However, the enzymatic treatment for human OA articular cartilage was failed on account of excessive cell aggregates, and more detailed work should be carried out in the future.

## Methods

### Preparation of the enzymatic solution

Media 199 (ThermoFisher Scientific, Ref# 12350039) supplemented with 2% Fetal Bovine Serum (FBS, ThermoFisher Scientific, Ref# 10082147) was used as basic medium. STEMxyme1 (Worthington, Ref# LS004106), Dispase II (ThermoFisher Scientific, Ref# 17105041), Pronase (Roche, Ref# 10165921001), Collagenase type I (ThermoFisher Scientific, Ref# 17100017) and Collagenase type II (ThermoFisher Scientific, Ref# 17101015) were respectively dissolved in 1 × phosphate-buffered saline (PBS) buffer to a concentration of 10 mg/mL. Filter the solution with a 0.22- $\mu$ m membrane filter and store at -20°C. The enzymatic solution for NP was prepared by adding STEMxyme1, Dispase II and Pronase solutions into the basic medium to the concentration of 1 mg/mL, 1 mg/mL and 2 mg/mL, respectively. For ossifying posterior longitudinal ligaments, STEMxyme1, Dispase II and Collagenase type I solutions were added into the basic medium to the same concentration of 1 mg/mL. Similarly, STEMxyme1, Dispase II and Collagenase type II solutions were added into the basic medium to the same concentration of 1 mg/mL, which was used in single-cell dissociation from OA articular cartilage. All enzymatic solutions must be freshly prepared each time.

### Human samples

The degenerated degree of intervertebral disc was assessed according to the modified Pfirrmann grading system using preoperative magnetic resonance imaging (MRI). The OPLL diagnosis was confirmed by X-ray, computed tomography (CT) and MRI. The severity of knee OA was evaluated based on Kellgren-Lawrence (K-L) scoring system. All human samples were hold in normal saline in ice-water bath as soon as resected during surgery and delivered to the following treatment in one hour. Cartilage was shaved from the underlying bone and cut into several pieces. All specimens were washed 3-5 times using ice-cold 1 × PBS buffer to remove the peripheral blood and debris.

### Isolation single cells

Firstly, the specimens were added 0.3mL of the enzymatic solution on the surface and minced into pieces of about 2 mm<sup>3</sup>. Then each 100mg specimen was added with 10mL enzymatic solution with agitation (180 rpm) at 37°C for 20 mins. Transiently centrifuge at 300g for 10 secs, transfer the supernatant cell suspension into a new tube and keep the residual tissue fragments at 4°C temporarily. Next, centrifuge the cell suspension at 300g for 5 mins. The supernatant enzymatic solution was added back into the tissue fragments for second digestion and the collected cell pellet was dispersed in 5 mL cell staining buffer (Biolegend, Ref# 420201). Keep the dispersed cells at 4°C temporarily. Repeat the above

operations for 3-5 times, and collect all cells after filtration through a 40µm filter into a collection tube. In this method, timely collection of dispersed cells would protect them from excessive digestion, and cyclic digestion would raise the cell yield. Wash with cell staining buffer for 3 times and resuspend the collected cells in 1 mL cell staining buffer for following operations. The residual tissue fragments were observed under stereo microscope (Olympus, SZX7).

### **Cell counting and quality control**

Cell counting was performed both manually and automatically (JIMBIO, JIMBIO CL). Hoechst 33342 (Invitrogen, Ref# R37605), calcein AM (Invitrogen, Ref# C1430) and propidium iodide (PI, Invitrogen, Ref# P3566) were added in 100µL cell suspension and incubated for 15 mins at room temperature in dark. Observe under fluorescence microscope.

### **Construction of scRNA-seq libraries and quality control**

The construction of scRNA-seq libraries was strictly performed according to the instructions. Briefly, qualified samples were identified as containing more than 85% viable cells, less than 10% doublets and no cell aggregates. Two of the qualified samples were delivered to scRNA-seq library construction and sequencing. Approximately 10,000 cells per sample were loaded onto 10X Genomics Chromium 3' Single Cell Gene Expression Solution v3 micro fluidics chip (10X Genomics, Ref# PN-2000060) to generate Gel Beads-in-emulsion (GEM) with barcodes. After break of the GEMs, reverse transcription was applied to generate the first-strand cDNA with purification using Silane magnetic beads. And then, barcoded and full-length cDNA was amplified via PCR to generate sufficient mass for library construction. Enzymatic fragmentation and size selection were used to optimize the cDNA amplicon size. After sequential add with P5, P7, a sample index and TruSeq Read 2, the libraries were constructed. The quality of the scRNA-seq libraries was analyzed by Agilent Bioanalyzer 2100 before Next-generation sequencing.

### **Statistical analysis**

All the statistical analysis was performed by SPSS 16.0 statistical software. Student's t-test was utilized. Quantitative data was presented as means  $\pm$  SD. P-value of less than 0.05 was considered to be statistically significant.

## **Abbreviations**

scRNA-seq: Single-cell RNA-sequencing

snRNA-seq: Single-nucleus RNA-sequencing

the S-D solution: 1 mg/mL STEMxyme1 (Worthington, Ref# LS004106) and 1 mg/mL Dispase II (ThermoFisher Scientific, Ref# 17105041) supplemented in Media 199 with 2% FBS

MACS: Magnetic-activated cell sorting

FACS: Fluorescence-activated cell sorting

PI: Propidium iodide

NP: Nucleus pulposus

OA: Osteoarthritis

OPLL: Ossification of the posterior longitudinal ligament

GEM: Gel Beads-in-emulsion

## **Declarations**

### **Ethics approval and consent to participate**

The study protocol was reviewed and approved by the institutional review board and ethics committee of the Seventh Affiliated Hospital of Sun Yat-sen University in Shenzhen, China (No. 2019SYSUSH-031), and written informed consents were obtained from all patients before employing the resected tissues in this study.

### **Consent for publication**

Not applicable.

### **Availability of data and material**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare no conflict of interests.

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## Authors' contributions

MG and ZZ conceived and designed the study; MG, PG and XL performed experiments with help from PZ, ZH and LW; SL and ZZ supervised the project; MG and PG wrote the manuscript. All authors have read and approved the final version of the manuscript.

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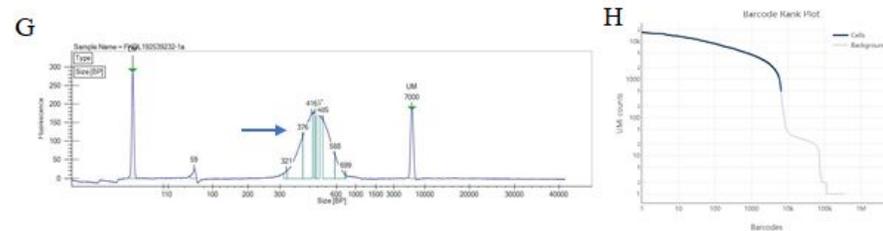
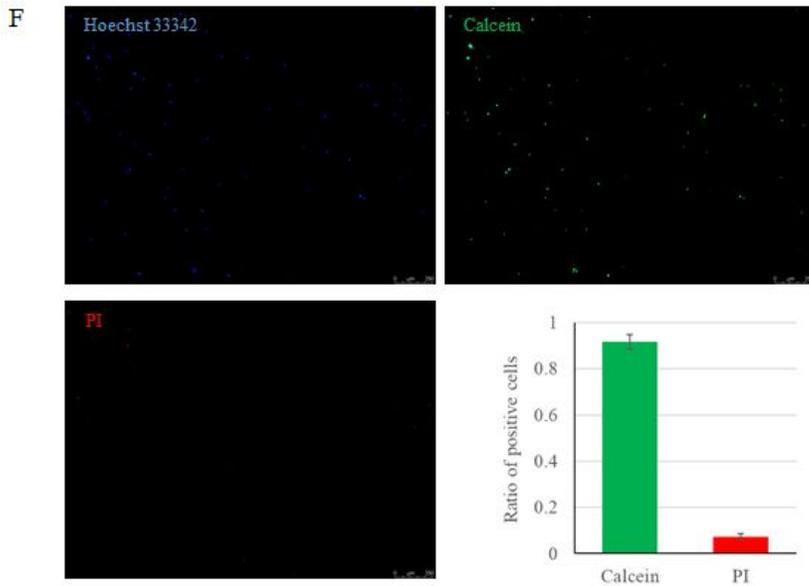
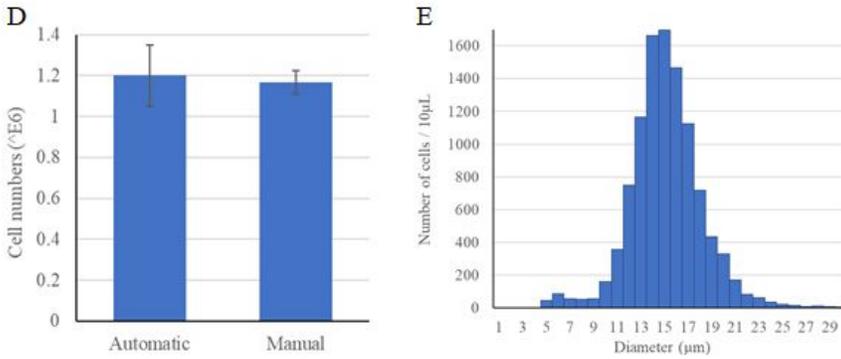
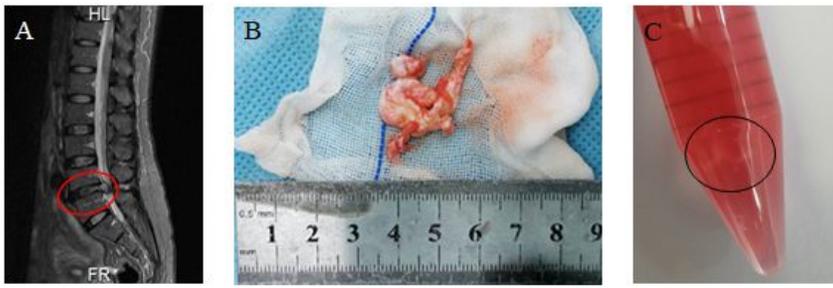
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## Figures



**Figure 1**

Isolation of single cells from degenerated nucleus pulposus (NP) and Single-Cell RNA Sequencing (scRNA-seq). A. Lumbar disc degeneration was shown on T2-weighted (T2-WI) Magnetic Resonance Imaging (MRI). The red circle marked the degenerated disc. B. Gross appearance of the degenerated NP obtained from surgery. C. Gross appearance of the cell suspension after 3-5 cycles of enzymatic treatment. Some white floccule residuals were labeled by black circle. D. Total cell numbers were counted

automatically and manually, respectively. E. The isolated cell diameter distribution histogram. F. The live/dead immunofluorescence staining and quantitative analysis. G. Bioanalyzer trace of the scRNA-seq library showed the size distribution between 300 and 600 bp (blue arrow). H. The barcode-rank plot displayed gene expression counts after sequencing.

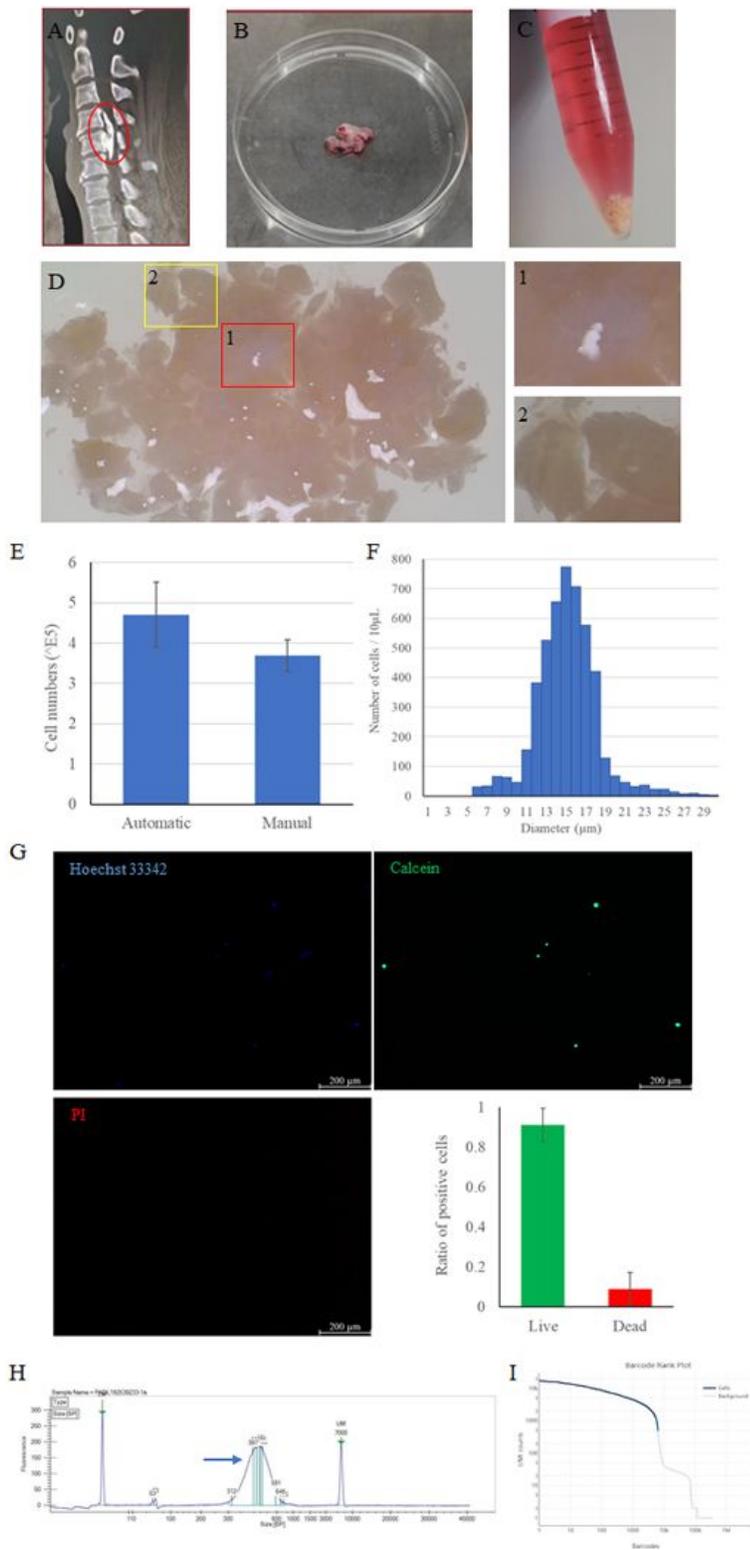


Figure 2

Isolation of single cells from ossifying posterior longitudinal ligament and scRNA-seq. A. Ossification of posterior longitudinal ligament (OPLL) was shown on the three-dimensional reconstruction of computerized tomography (CT) image (red circle). B. Gross appearance of the ossifying posterior longitudinal ligament harvested during surgery. C. Gross appearance of the cell suspension and residuals after 3-5 cycles of enzymatic digestion. D. Stereo microscope images showed the residuals. The red box enclosed some white soft viscous residuals magnified in the image labeled 1. And the yellow box marked part of the bone fragments magnified in the image labeled 2. E. Total cell numbers were counted automatically and manually, respectively. F. The isolated cell diameter distribution histogram. G. The live/dead immunofluorescence staining and quantitative analysis. H. Bioanalyzer trace of the scRNA-seq library showed the size distribution between 300 and 600 bp (blue arrow). I. The barcode-rank plot displayed gene expression counts after sequencing.

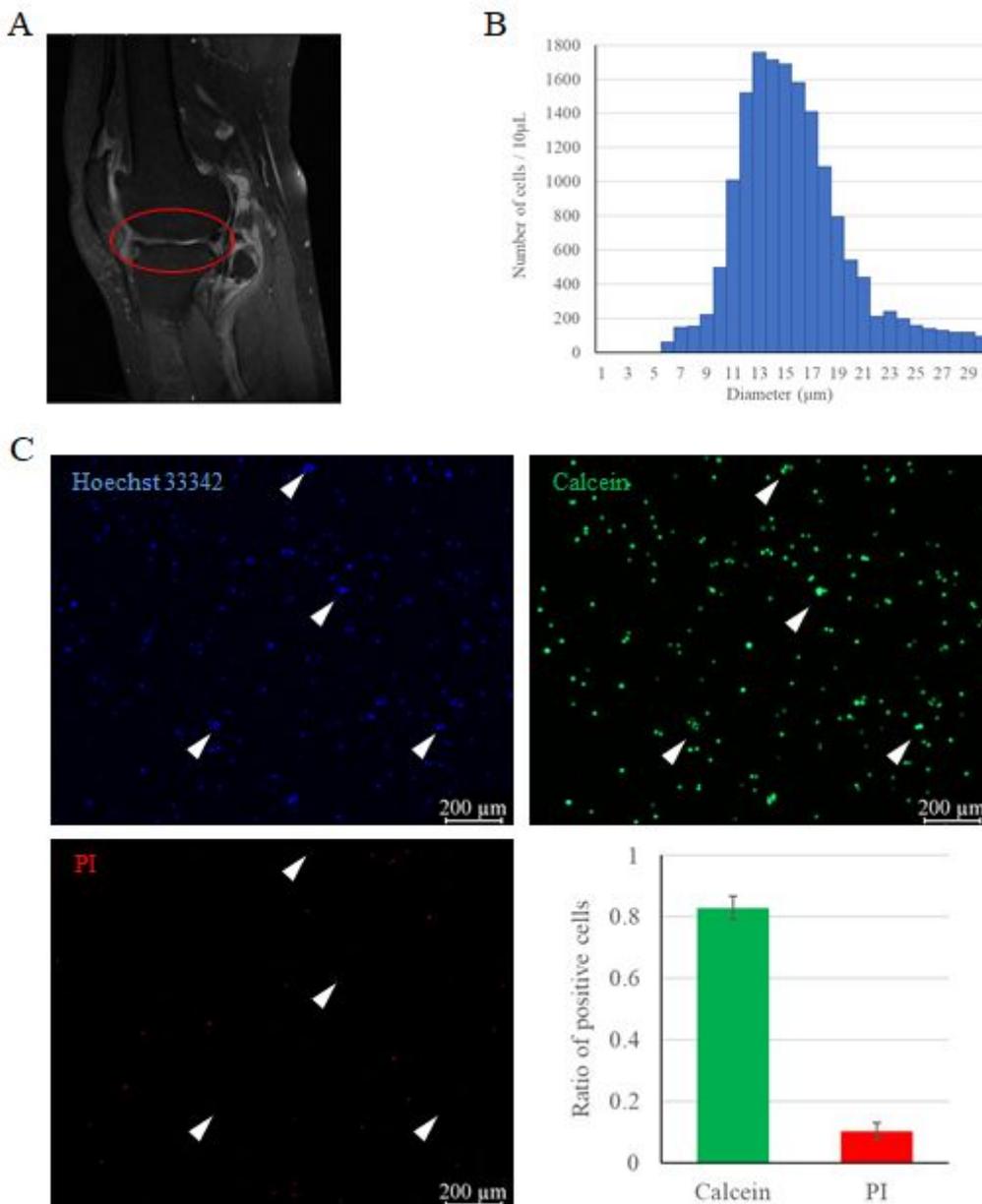
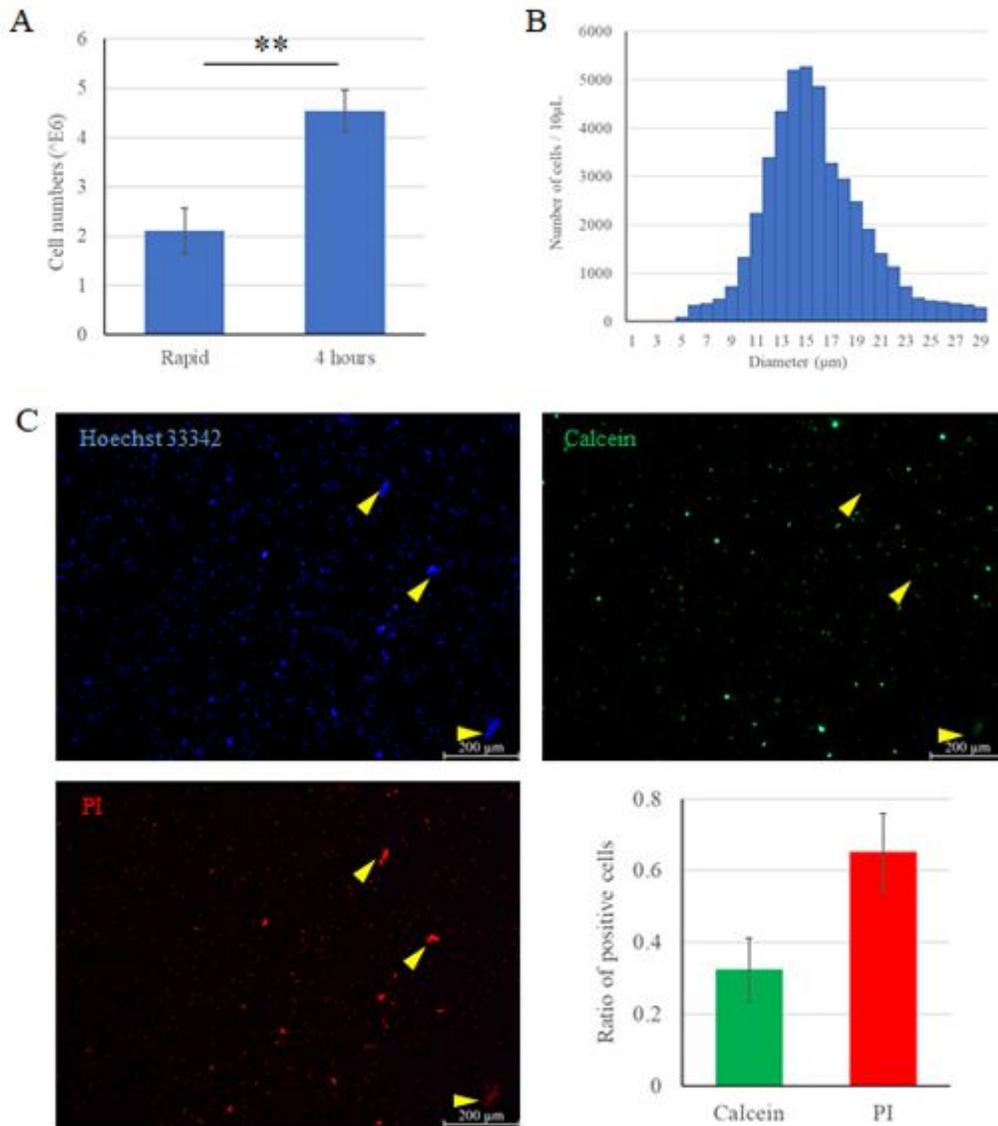


Figure 3

Rapid isolation of single cells from knee articular cartilage with osteoarthritis (OA) and the quality control. A. OA was characterized on T2-weighted imaging (T2-WI). The affected region was circled in red. B. The isolated cell diameter distribution histogram. C. The live/dead immunofluorescence staining and quantitative analysis. White arrowheads highlighted the cell aggregates, which showed Hoechst 33342+, Calcein+ and PI-.



**Figure 4**

Prolonged isolation of single cells from knee articular cartilage with OA and the quality control. A. The total cell numbers counted automatically in both rapid and prolonged enzymatic treatment groups. Prolonged and continuous digestion would significantly increase cell yield ( $P < 0.01$ ). B. The isolated cell diameter distribution histogram. C. The live/dead immunofluorescence staining and quantitative analysis. Yellow arrowheads highlighted the cell aggregates, which showed Hoechst 33342+, Calcein- and PI+.