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Rapid Isolation of Single Cells from Mouse and Human Teeth

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TITLE:

Rapid Isolation of Single Cells from Mouse and Human Teeth

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KEYWORDS:

Single-cell isolation, mouse incisor, dental development, tooth, tissue processing

SUMMARY:

The current protocol presents a fast, efficient, and gentle method for isolating single cells suitable for single-cell RNA-seq analysis from a continuously growing mouse incisor, mouse molar, and human teeth.

ABSTRACT:

Mouse and human teeth represent challenging organs for quick and efficient cell isolation for single-cell transcriptomics. The dental pulp tissue, rich in the extracellular matrix, requires a long and tedious dissociation process that is typically beyond the reasonable time for single-cell transcriptomics. For avoiding artificial changes in gene expression, the time elapsed from euthanizing an animal until the analysis of single cells needs to be minimized. This work presents a fast protocol enabling to obtain single-cell suspension from mouse and human teeth in an excellent quality suitable for scRNA-seq (single-cell RNA-sequencing). This protocol is based on accelerated tissue isolation steps, enzymatic digestion, and subsequent preparation of final single-cell suspension. This enables fast and gentle processing of tissues and allows using more animal or human samples for obtaining cell suspensions with high viability and minimal transcriptional changes. It is anticipated that this protocol might guide researchers interested in performing the scRNA-seq not only on the mouse or human teeth but also on other extracellular matrix-rich tissues, including cartilage, dense connective tissue, and dermis.

INTRODUCTION:

Single-cell RNA sequencing is a powerful tool for deciphering *in vivo* cell population structure, hierarchy, interactions, and homeostasis[1,2]. However, its results strongly depend on the first step of this advanced analysis - the preparation of a single-cell suspension of perfect quality out of the complex, well-organized tissue. This encompasses keeping cells alive and preventing unwanted, artificial changes in gene expression profiles of the cells[3,4]. Such changes might lead to the inaccurate characterization of population structure and widespread misinterpretation of the collected data.

Specific protocols for the isolation out of a wide range of tissues have been developed[5–8]. They usually employ mechanical dissociation in combination with further incubation with various proteolytic enzymes. These typically include trypsin, collagenases, dispases, papain[6–9], or commercially available enzyme mixtures such as Accutase, Tryple, etc.[5]. The most critical part affecting the transcriptome quality is enzymatic digestion. It was shown that prolonged incubation with enzymes at 37 °C influences the gene expression and causes the upregulation of many stress-related genes[10–13]. The other critical parameter of the isolation process is its overall length, as it has been shown that cell transcriptomes change after the death of the individual[14]. Together, an efficient protocol is developed for gentle isolation of single cells from murine and human teeth faster than other, previously utilized protocols for isolation of cells from complex tissues[5,6,9,11,13,15,16].

This protocol presents how to quickly dissect soft tissue from the hard tooth and prepare a single-cell suspension suitable for scRNA-seq. This method employs only one centrifugation step and minimizes the effect of unwanted transcriptional changes by reducing the tissue handling and digestion time and keeping the tissue and cells at 4 °C most of the time. The procedure showcases the isolation of cells from mouse incisors, molar, and human wisdom teeth as an example, but principally works for other mouse and human teeth. The complete protocol is schematically visualized in **Figure 1**. This protocol has been recently used to generate a dental cell type atlas obtained from mouse and human teeth[1].

PROTOCOL:

All animal experiments were performed according to the International and local regulations and approved by the Ministry of Education, youth and sports, Czech Republic (MSMT-8360/2019-2; MSMT-9231/2020-2; MSMT-272/2020-3). This protocol was tested with both male and female wildtype C57BL/6 and CD-1 mice and with genetically modified Sox10::iCreER^{T2} mice[17] (combined with various reporter systems) on a C57BL/6 background. Experiments with human samples were performed with the approval of the Committees for Ethics of the Medical Faculty, Masaryk University Brno & St. Anne´s Faculty Hospital in Brno, Czech republic.

1. Experimental set-up and preparation of solutions

1.1. Instrument set-up

1.1.1. Cool down the centrifuge to 4 °C.

1.1.2. Heat the incubation chamber to 37 °C.

1.1.3. Start the Fluorescence-activated cell sorting (FACS) machine and set all the temperatures of the sorter (including collection tube holder) to 4 °C. Perform the instrument quality control, set the drop delay.

1.1.4. Set the preliminary gating strategy and perform the test sorting.

NOTE: When using FACS, use the 100 µm nozzle.

1.2. Prepare the solutions (steps 1.2.1–1.2.3).

1.2.1. Wash solution: Prepare fresh 2% FBS (Fetal Bovine Serum) in HBSS (Hanks' Balanced Salt Solution).

1.2.2. Digestion mixture: Prepare fresh collagenase P (3 U/mL) fully dissolved in HBSS.

1.2.3. Optional: Prepare fresh HBSS + BSA (0.04%) and chill analytical grade methanol in a -20 °C freezer for storing single-cell suspension at -80 °C.

NOTE: Step 1 needs to be performed before the start of the experiment. The composition of the utilized solutions is summarized in **Supplementary Table 1**.

2. Preparation of experimental animal/s and human tooth

2.1. Prepare the experimental animals (mouse).

2.1.1. Euthanize the mouse according to the local regulations; e.g. by anaesthetics overdose as described previously[1].

CAUTION: Regulations for humane euthanizing of experimental animals varies locally. Always follow valid local regulations.

2.1.2. Immediately proceed to the tissue dissection step (step 3).

NOTE: If the tissue from the experimental animals cannot be dissected immediately (e.g., because of transfer from animal housing facility), place the experimental animals on ice and perform tissue dissection as soon as possible. To obtain more cells from mouse molar pulps, use younger (6 weeks and less) animals. With increasing age, the size of dental pulp decreases. Mouse incisors mostly keep their structure with increasing age so animals of various ages can be used for tissue dissection.

2.2. Prepare the human tooth

NOTE: Human teeth were extracted for a clinically relevant reason. Every diagnosis was treated individually, and an experienced dental surgeon always performed the tooth extraction.

2.2.1. Put the freshly extracted tooth immediately into a 50 mL tube with ice-cold HBSS and keep the tube on ice until further processing.

NOTE: Using retained wisdom teeth from patients until age 25–30 is recommended for the highest cell yield.

3. Tissue dissection

3.1. Hold the experimental animal behind its head, looking on the ventral aspect of its head so that the tail points away.

3.2. Using small, sharp scissors, quickly remove the skin from the mandible to expose the mandibular arch, the soft tissue between each half of the mandibles, and the adjacent facial muscles.

3.2.1. Make a deep cut from each side of the mandible; firstly, through m. masseter along the buccal side of the mandible up to the temporomandibular joint, and then along the inner part of each half of the mandible through the base of the oral cavity (see **Supplementary Figure 1**).

3.3. Cut all the muscles and ligaments along the mandible up to the temporomandibular joint from both outside and inside of the oral cavity.

NOTE: Avoid cutting bones. This might damage the most apical part of the incisor.

3.4. Grasp the mandible using bent tip tweezers and remove it. Then, split the dissected mandible into two halves with scissors by cutting through mandibular symphysis (see **Supplementary Figure 1**).

3.5. Use an industrial low lint wipe to chafe the remaining soft tissue from each half of the mandible. After both parts of the mandible are cleaned, place them into a pre-prepared Petri dish with ice-cold HBSS.

NOTE: From this point forward, work on ice. Further dissection of mouse incisors and molars is performed under a stereomicroscope with a black background.

3.6. Mouse mandibular incisors

3.6.1. For the dissection of mandibular incisors, remove the alveolar ridge with all three molars and transversally crack the mandibular arch in the place corresponding to the position between

the first and second molar.

NOTE: A sharp scalpel blade no. 11 and tweezers are used to perform this step (see **Table of Materials**).

3.6.2. Carefully pull the incisor out of the rest of the dental socket.

NOTE: If successful, the extracted incisor will contain intact apical parts, including epithelial tissue with complete cervical loops.

3.6.3. If needed, remove the remaining fragments of bone still attached to the incisor with tweezers and a scalpel.

3.6.4. Place the dissected incisors into fresh, ice-cold HBSS and dissect the tissue of interest: cervical loop, dental pulp, or other parts of the tooth.

3.6.5. Place the dissected soft tissue into a droplet of fresh, ice-cold HBSS in the middle of a 10 cm Petri dish. Keep on ice.

3.7. Mouse mandibular molars

3.7.1. For dissecting mandibular molars, completely remove the alveolar ridge from the rest of the mandible using a scalpel blade.

3.7.2. Move the dissected alveolar crest into fresh, ice-cold HBSS in a Petri dish and carefully remove all the remaining fragments of alveolar bones attached to the roots.

3.7.3. Place the dissected molars without alveolar bone into fresh, ice-cold HBSS. To expose the pulp, start to remove parts of the dentin from the apical side using fine, sharp tip tweezers until you reach the pulp cavity.

3.7.4. Once the pulp cavity is reached, carefully dissect dental pulp using a pair of sharp tip tweezers and place the soft tissue of the dental pulp into a droplet of fresh, ice-cold HBSS in the middle of a 10 cm Petri dish kept on ice.

NOTE: Since mouse molar pulps are extremely small, adapt magnification on the stereomicroscope accordingly.

3.8. Human tooth

3.8.1. Wash human tooth once again in ice-cold HBSS to remove the remaining blood.

3.8.2. Place the tooth into three thick-walled sterile plastic bags and use a cast iron benchtop engineer's vise to crack the tooth. Use vise jaws with a flat surface to avoid penetrating the bags.

3.8.3. Slowly tighten the vise until you hear the tooth cracking; remove it from the bags and place it into fresh, ice-cold HBSS in a Petri dish.

3.8.4. Using two tweezers, take out the dental pulp, clean it from all the remnants of hard tissue and place it into one droplet of ice-cold HBSS in the middle of a 10 cm Petri dish kept on ice.

CAUTION: Human tissue might potentially be infectious. When working with human tissue, use protective equipment to avoid direct contact with the tissue.

4. Preparation of single-cell suspension

4.1. Prepare a 15 mL tube with 2.5 mL of digestion mixture composed of Collagenase P (3 U/mL) fully dissolved in HBSS. Keep the solution on ice until use.

NOTE: Always use freshly prepared Collagenase P; do not freeze the aliquots. Collagenase P activity varies from batch-to-batch. Check the activity of your batch before diluting. Collagenase P is activated by calcium, whose amount is already sufficient in the lyophilized powder. Therefore, it is unnecessary to enrich the enzyme mix with calcium. Collagenase P is not inactivated by FBS but can be inactivated by chelating agents (e.g., Ethylenediaminetetraacetic - EDTA). Stopping the dissociation process is ensured by diluting the Collagenase P in Wash solution (2% FBS in HBSS). It has been shown that adding FBS increases cell viability and the final number of cells after sorting[18].

4.2. Using a round-shaped scalpel blade no. 10, cut the tissue in all directions into the smallest possible pieces. Reaggregate the tissue pieces in the middle of the droplet and repeatedly cut the tissue aggregates using a round-shaped (No. 10) scalpel blade. Repeat this process several times until the material is sufficiently minced.

4.3. Transfer the shredded tissue pieces using a 1 mL pipette tip into the prepared digestion mixture.

NOTE: In the case of a larger number of animals being processed at once, split the tissue into several tubes with Collagenase P. The amount is approximately the pulp from 10 incisors for one tube. In the case of molars, where pulps are much smaller, the number of processed pulps can be increased adequately. When using human teeth, use a maximum of 2–3 pulps per tube, depending on the pulp size.

4.4. Place the tube into a 37 °C preheated incubator with a shaker. Tilt the tube inside the shaker to an angle of 60° and set the speed to 150–200 rpm to ensure constant suspension movements inside the tube.

4.5. Vigorously triturate the suspension every 3–4 min with a 1 mL pipette tip to disintegrate all the clumps.

NOTE: With time, the clumps will become smaller and softer until they almost disappear.

4.6. In total, incubate for 15–20 min. At the end of the incubation, triturate for the last time, and then slowly add ice cold Wash solution to a final volume of 12 mL.

4.7. Remove the remaining clumps or pieces of calcified tissue filter cell suspension using a 50 µm cell strainer.

4.8. Take 10–20 µL of the filtered cell suspension and count the cells during centrifugation using a cell counting chamber (Hemocytometer). Centrifuge for 5 min at 300 x *g* at 4 °C. Remove the supernatant using a 10 mL serological pipette.

NOTE: If the number of cells is limited that cannot be counted in diluted suspension, skip the cell counting and use all the cells for further processing.

4.9. Optional: Proceed for fixation and storage.

4.9.1. Re-suspend the pellets (up to 10^7 cells) in 100 uL of HBSS + BSA (0.04%).

4.9.2. Add 400 µL of ice-cold methanol and mix slowly.

4.9.3. Incubate the cell suspension for 15 min on ice.

4.9.4. Store at -80 °C until processed (no longer than 1 month).

4.10. Resuspend the pellet in the Wash solution. Aim for 700–1200 cells/µL of the Wash solution.

4.11. Keep the tube on ice until further processing.

NOTE: If necessary, perform the fixation and storage at -80 °C. However, immediate processing of the cell suspension for scRNA-seq is recommended. Further steps will depend based on the single-cell RNA seq protocol. When the scRNA-seq protocol uses a microfluidic system (some sc-RNA-seq companies do), load the cells based on the manufacturer's guidelines either directly or after cell sorting.

4.12. For FACS, proceed to step 5.

5. Fluorescence-activated cell sorting (FACS)

5.1. Before sorting, prepare the cell sorting instrument.

5.1.1. Cool down the whole system to 4 °C, perform the instrument quality control, set the drop

delay and the voltage of the deflection plates. Put collection tubes in the collection tube holder.

NOTE: To avoid additional centrifugation steps resulting in an inevitable cell loss, sort cells into a microtube (1.5 mL) with a small amount (25–50 µL) of the Wash solution.

5.2. Optional: perform viability staining before FACS.

5.2.1. Add Propidium iodide into cell suspension before FACS to a final concentration 0.5 µg/mL and incubate for 15 min at 4 °C.

5.3. Load the sample into the cell sorter. Set a strict gating strategy to remove cell debris, doublets, and dead cells. Sort the cells into a prepared tube or multi-well plates. An example of a gating strategy is shown in **Figure 2**.

NOTE: To minimize manipulation steps and accelerate the protocol, applying a strict gating strategy is recommended (suggested in **Figure 2**) rather than using a viability staining. To separate the immune cells from the isolated population, CD45 antibody staining can be performed. To perform this, resuspend cell suspension in 100 µL of staining solution (PBS + 2% FBS + anti-CD45-APC conjugated antibody 1/100 dilution). Incubate for 15 min on ice protected from light and perform FACS directly.

REPRESENTATIVE RESULTS:

Exemplary isolation of single cells was performed from two mandibular incisors from one 6-week-old C57BL/6 mouse male. Following this protocol, a single-cell suspension was prepared, and subsequently, single-cell sequencing was performed. The prepared single-cell suspension was analyzed and sorted using FACS (**Figure 2**). Firstly, the FSC-A (forward scatter, area) and SSC-A (side scatter, area) plotting was applied, and an appropriate gating strategy was used to select a population with expected size and granularity to filter our cell debris and cell doublets or aggregates (**Figure 2A**). This selected population (P1), counting 38% of all events, was further used, and FSC-A and FSC-H (forward scatter, height) parameters were applied to remove the remaining cell doublets (**Figure 2B**). The population without cell doublets (P2) counting 95% of P1 can be subsequently used for scRNA-seq. Alternatively, additional gating can be used to select the population of interest (e.g., expression of fluorescent proteins or live/dead staining). To check the number of live/dead cells in the final suspension, the PI (propidium iodide) staining was performed (**Figure 2C**). The P3 population containing PI^- (living) cells was 98.4% out of the parent P2 population and 35.5% out of the total events. The total number of filtered out, dead (PI^+) cells was 1887.

The final number of cells obtained without viability staining suitable for RNA-seq (P2) counted 118,199 cells from two mouse incisor pulps. This means the number of almost 60,000 living cells from one mandibular incisor.

To clarify the number of immune cells in the final single-cell suspension, two approaches were used. Firstly, the CD45 antibody staining and subsequent FACS analysis were used. As a

complementary method, the total number of immune cells (CD45+) in scRNA-seq data was analysed. FACS analysis showed 14.44% of CD45+ cells (13.20% alive and 1.24% dead)(**Figure 3A**). Analysis of scRNA-seq data showed 10.90% of CD45-expressing cells (**Figure 3B**). The decrease of CD45+ cells in scRNA-seq data can be caused by additional thresholding during scRNA-seq analysis.

These representative data on the example of mouse incisor show that the given protocol in combination with strict gating strategy is efficient in obtaining a high number of cells out of a single mouse tooth without the necessity of additional use of viability staining. The ratio of immune (CD45+) cells was minor (13.2%). Moreover, it was previously shown that the immune cells are essential in maintaining tooth homeostasis, so removing them from scRNA-seq analysis during the FACS would be counterproductive in some applications.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the protocol. Different steps, including temperature conditions and expected time, are represented to prepare single-cell suspension from mouse and human teeth.

Figure 2: Example of the gating strategy. FSC-A and SSC-A gating was used to produce the P1 gate, reflecting the cell population with expected size and granularity and filtering out the cell and extracellular matrix debris and most large events (**A**). Subsequently, the P1 population was plotted in FSC-H and FCS-A plot, which filtered out cell doublets (**B**). This P2 population was then analyzed for the presence of dead cells by propidium iodide (**C**). The number of events/cells per gate are represented in (**D**). (FSC-A – forward scatter, area; SSC-A – side scatter, area; FSC-H – forward scatter, height; PI – propidium iodide).

Figure 3: Quantification of the immune cells. Quantification of the immune cells was performed by FACS analysis of cells stained with anti-CD45 antibody and Live/Dead analysis using propidium iodide staining (**A**). Further quantification of immune cells was performed during scRNA-seq analysis (**B**). (CD45-APC – anti-CD45 allophycocyanin conjugated antibody; PI – propidium iodide; t-SNE – t-distributed stochastic neighbor embedding).

Supplementary Figure 1: Overview of the mandible dissection process. Dashed lines illustrate suggested cuts. TMJ – temporomandibular joint, m. masseter – musculus masseter.

Supplementary Table 1: The compositions of the solutions used in the study.

DISCUSSION:

Studying teeth and bones on the cellular or molecular level is generally challenging since cells forming these tissues are surrounded by different kinds of hard matrices[19]. One of the main goals for performing single-cell RNA-seq on dental tissue is the need to obtain cells of interest fast and without any artificial changes in their transcriptomes. To accomplish this, a highly efficient protocol suitable for isolating cells from mouse and human tooth pulps was developed, which

allows for quick generation of single-cell suspensions for all transcriptomic applications. This was ensured by fast tissue isolation, minimizing the steps of tissue and cell manipulations, and streamlining the mechanical and enzymatic digestion.

The most critical steps of this protocol are fast tissue processing and adequate single-cell suspension preparation[8,9]. A manual approach is used to obtain dental pulps without utilizing a dental drill or other heat-generating devices. Overheating may cause an artificial expression of heat shock proteins and other genes, ultimately leading to the analyzed gene expression patterns being unrepresentative of the original tissue[20]. Manual tissue harvesting may be a challenging step that will likely need some training beforehand. The pulp is then cut into small pieces and enzymatically digested at 37 °C. Except for the 15–20 min of enzymatic digestion, the whole protocol is performed at 4 °C. The tissue processing and especially the enzymatic digestion were minimized to the shortest possible time since more prolonged incubation at 37 °C can cause changes in gene expression patterns[10]. Mechanical removing of the dentin is recommended as much as possible before enzymatic digestion. Dentin and the pulp-attached predentin contain a large amount of collagen, and its excessive presence might decrease the effectiveness of the digesting solution. After being removed from the body (or death of organisms), it was shown that cells start to modify their gene expression patterns quickly[12]. Therefore, cell isolation and processing should be carried out as fast as possible. The current protocol reduces the processing time to 35–45 min from receiving the tissue (euthanizing animal) to preparing single-cell suspension.

One alternative modification of this technique is cell preservation for later use. This is achieved by methanol fixation. Methanol-fixed cell suspension can be stored for up to 1 month at -80 °C, as described in the protocol[21]. However, whenever possible, perform scRNA-seq directly, since it was shown that the single-cell data from methanol-fixed single-cell suspensions might suffer from increased expression of stress-related genes and contamination with ambient RNA[22]. This step might need additional modification according to the manufacturer's protocols.

Before the first application of this protocol, performing several validation steps are recommended to test the technique. From our experience, we suggest testing the aforementioned critical steps of the protocol. Additionally, we suggest testing the effectiveness of the collagenase P solution and testing the handling of the tissue dissociation step. Specifically, around the first 5 min after the initiation of collagenase P incubation, the pieces of tissue should aggregate together. This is a common situation. Aggregates are disintegrated every 3–4 min using a 1 mL pipette, and with increasing time, they should become smaller until barely visible.

Furthermore, it is recommended to perform cell counting in a cell counting chamber before centrifugation and before and after filtering to detect possible cell losses due to suboptimal supernatant removal. If the final single-cell suspension needs to be purified, FACS can be used. Cell sorting enables not only to remove debris or dead cells but importantly enables to enrich final suspension with fluorescently labeled cells[13,19]. To avoid shear stress or clogging of the cell sorter, a wide nozzle (85 µm or 100 µm) is used. This will further improve the viability of the sorted cells.

This technique was designed and tested on both mouse and human teeth. The major limiting factor is the small number of cells in the reduced dental pulps of the teeth of older mice (molars) and humans. Suppose a larger number of cells need to be obtained or cells from the teeth of older patients are to be acquired. One possible solution is to process a higher number of teeth and merge them into a single batch, subsequently processed as one sample.

Living cells of human dental pulp were firstly isolated more than twenty years ago using an enzyme mixture of collagenase I and dispase[23]. Since then, isolations of dental pulp cells became widely utilized, and several techniques have been used[5–8]. The critical significance of the method presented here is the adaption of all isolation steps to make the isolation fast and gentle to ensure the high quality of the final cell suspension for scRNA-seq. Higher cell yield can be obtained by more prolonged incubation with enzymes. This protocol provides an efficient solution for quickly obtaining single cells from mouse and human teeth of suitable quality for single-cell RNA-sequencing. This technique is expected to be widely used for other tissues or organisms with just slight technical modifications.

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DISCLOSURES:

Authors declare no conflicts of interest.

REFERENCES:

1. Krivanek, J. et al. Dental cell type atlas reveals stem and differentiated cell types in mouse and human teeth. *Nature Communications*. **11** (1), 4816 (2020).
2. Soldatov, R. et al. Spatiotemporal structure of cell fate decisions in murine neural crest. *Science*. **364** (6444) (2019).
3. Machado, L., Relaix, F., Mourikis, P. Stress relief: emerging methods to mitigate dissociation-induced artefacts. *Trends in Cell Biology*. **0** (0) (2021).
4. Nguyen, Q. H., Pervolarakis, N., Nee, K., Kessenbrock, K. Experimental considerations for single-cell RNA sequencing approaches. *Frontiers in Cell and Developmental Biology*. **6** (2018).
5. Chiba, Y. et al. Single-cell RNA-sequencing from mouse incisor reveals dental epithelial cell-type specific genes. *Frontiers in Cell and Developmental Biology*. **8** (2020).
6. Debnath, S. et al. Discovery of a periosteal stem cell mediating intramembranous bone formation. *Nature*. **562** (7725), 133–139 (2018).
7. Kanton, S., Treutlein, B., Camp, J. G. Chapter 10 - Single-cell genomic analysis of human cerebral organoids. *Methods in Cell Biology*. **159**, 229–256 (2020).

8. Price, F. D. et al. Inhibition of JAK-STAT signaling stimulates adult satellite cell function. *Nature Medicine*. **20** (10), 1174–1181 (2014).
9. van den Brink, S. C. et al. Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations. *Nature Methods*. **14** (10), 935–936 (2017).
10. O'Flanagan, C. H. et al. Dissociation of solid tumor tissues with cold active protease for single-cell RNA-seq minimizes conserved collagenase-associated stress responses. *Genome Biology*. **20** (1), 210 (2019).
11. van Velthoven, C. T. J., de Morree, A., Egner, I. M., Brett, J. O., Rando, T. A. Transcriptional profiling of quiescent muscle stem cells in vivo. *Cell Reports*. **21** (7), 1994–2004 (2017).
12. Miyawaki-Kuwakado, A. et al. Transcriptome analysis of gene expression changes upon enzymatic dissociation in skeletal myoblasts. *Genes to Cells*. **26** (7), 530–540 (2021).
13. Mattei, D. et al. Enzymatic dissociation induces transcriptional and proteotype bias in brain cell populations. *International Journal of Molecular Sciences*. **21** (21), 7944 (2020).
14. Ferreira, P. G. et al. The effects of death and post-mortem cold ischemia on human tissue transcriptomes. *Nature Communications*. **9** (1), 490 (2018).
15. He, W., Ye, J., Xu, H., Lin, Y., Zheng, Y. Differential expression of α6 and β1 integrins reveals epidermal heterogeneity at single-cell resolution. *Journal of Cellular Biochemistry*. **121** (3), 2664–2676 (2020).
16. Machado, L. et al. In situ fixation redefines quiescence and early activation of skeletal muscle stem cells. *Cell Reports*. **21** (7), 1982–1993 (2017).
17. Laranjeira, C. et al. Glial cells in the mouse enteric nervous system can undergo neurogenesis in response to injury. *The Journal of Clinical Investigation*. **121** (9), 3412–3424 (2011).
18. Song, X. et al. Improved strategy for jet-in-air cell sorting with high purity, yield, viability and genome stability. *FEBS Open Bio*. **11** (9), 2453–2467 (2021).
19. Greenblatt, M. B., Ono, N., Ayturk, U. M., Debnath, S., Lalani, S. The unmixing problem: A guide to applying single-cell RNA sequencing to bone. *Journal of Bone and Mineral Research*. **34** (7), 1207–1219 (2019).
20. Charlebois, D. A., Hauser, K., Marshall, S., Balázsi, G. Multiscale effects of heating and cooling on genes and gene networks. *Proceedings of the National Academy of Sciences*. **115** (45), E10797–E10806 (2018).
21. Chen, J. et al. PBMC fixation and processing for Chromium single-cell RNA sequencing. *Journal of Translational Medicine*. **16** (1), 198 (2018).
22. Denisenko, E. et al. Systematic assessment of tissue dissociation and storage biases in single-cell and single-nucleus RNA-seq workflows. *Genome Biology*. **21** (1), 130 (2020).
23. Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., Shi, S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. **97** (25), 13625–13630 (2000).

Figure 1

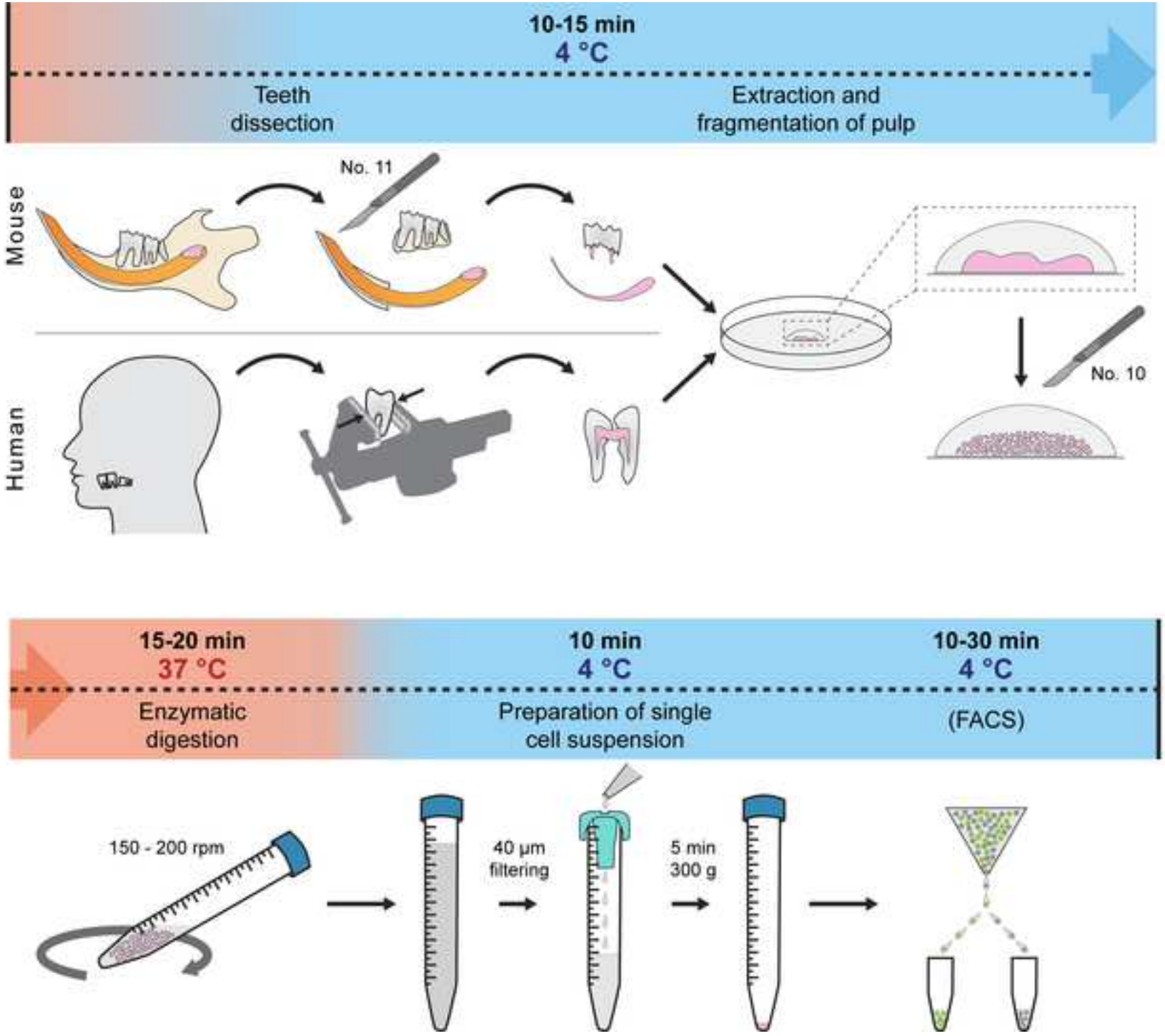
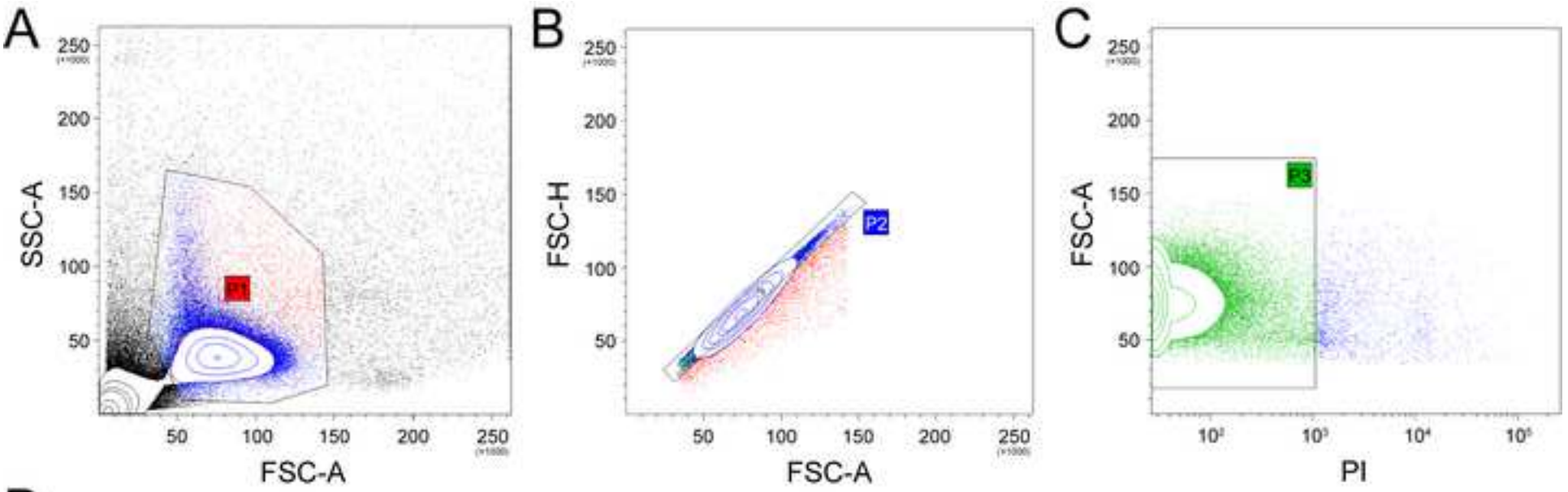


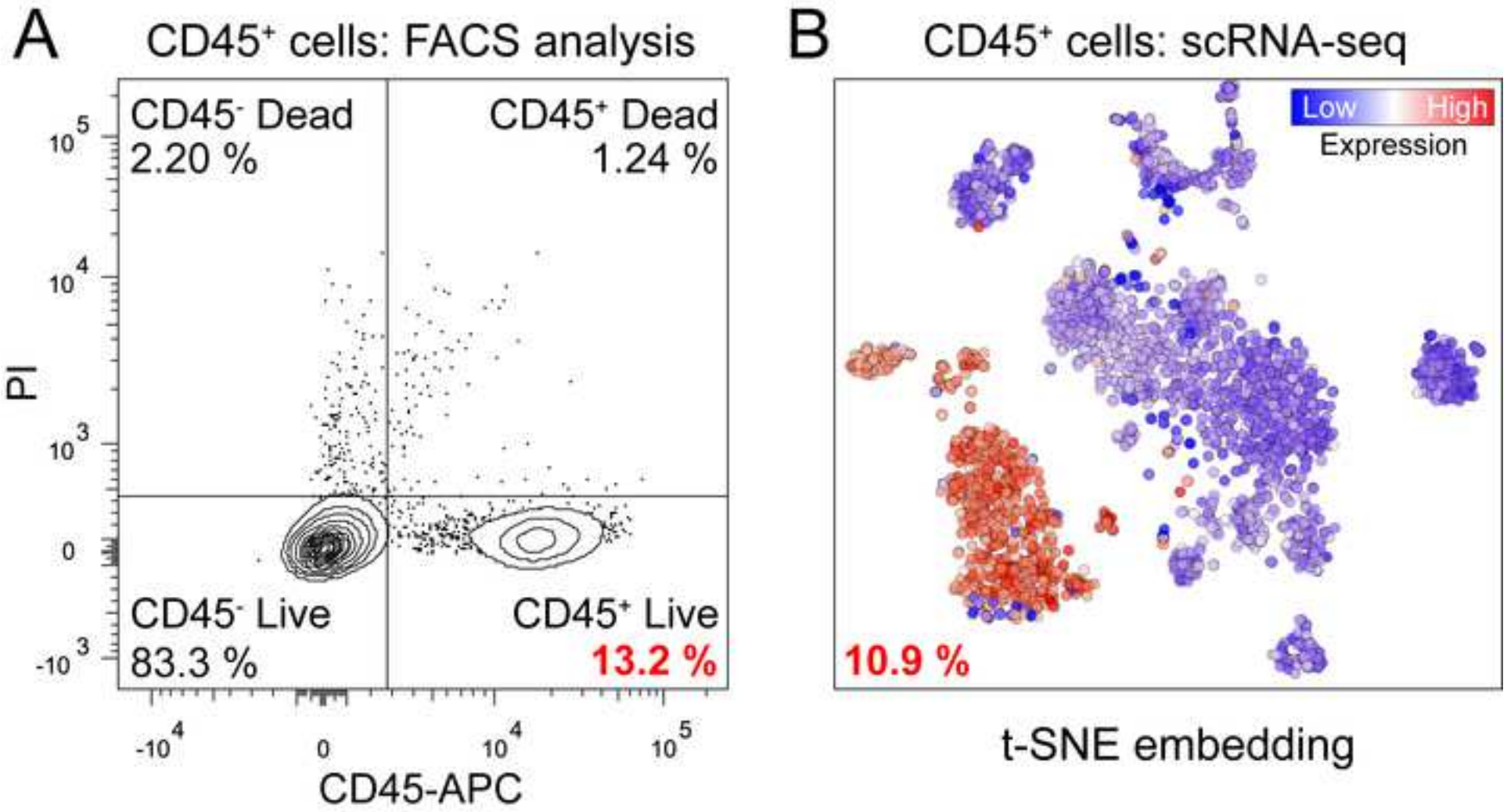
Figure 2



D

Population	#Events	%Parent	%Total
All Events	327,318	####	100.0
P1	124,380	38.0	38.0
P2	118,199	95.0	36.1
P3	116,312	98.4	35.5

Figure 3



Dear Editorial board,

we are grateful for reading our manuscript and for very constructive comments which helped to increase the overall quality of this methodological manuscript. Below you can find answers to all your comments addressed one by one.

Kind regards,

Jan Krivanek

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We performed proofreading and corrected grammar issues.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Now, wherever possible, we modified our text according to this comment.

3. Please ensure that abbreviations are defined at first usage.

We revised our text and checked used abbreviations.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

We changed the format of references according to this comment.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. For example, please remove 10x Genomics from Line 203. All commercial products should be sufficiently referenced in the Table of Materials (including reagents, instruments, software, etc.). Please sort the Materials Table alphabetically by the name of the material.

We removed "10x genomics" now and updated the Table of Materials.

6. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal and human care guidelines of your institution.

We added the following Ethics statement in the very beginning of our protocol in the revised version of our manuscript:

"All animal experiments were performed according to international and local regulations and approved by Ministry of education, youth and sports, Czech Republic (MSMT-8360/2019-2; MSMT-9231/2020-2; MSMT-272/2020-3)."

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

In the revised version of our manuscript, we added several clarifications and more detailed description of the protocol as it is discussed further.

8. Please add more details to your protocol steps:

Step 1.1: Please specify the age/gender/strain of the mouse used.

We moved our previous note (bellow point 2.8.4) to step 1.1 and modified it according to this comment as follows:

"To obtain a higher number of cells from mouse molar pulps use younger (6 weeks and less) animals. With increasing age the size of dental pulp decreases. The same situation applies to human teeth. For the highest yield of cells, we recommend using retained wisdom teeth from patients until the age of 25-30. Mouse incisors mostly keep their structure with increasing age so animals of various ages can be used for tissue dissection. This protocol was tested with both male and female wildtype C57BL/6 and CD-1 mice as well as with genetically modified (Cre-loxP combined with various reporter systems) mice on a C57BL/6 background. "

Step 2.2: Please quantify the cut to be made.

We updated description of this step as following:

"... Make a deep cut from each side of the mandible. Firstly, through m. masseter along the buccal side of the mandible up to the temporomandibular joint and then along the inner part of each half of the mandible through the base of the oral cavity."

Step 3.9: Please clarify how the cells are counted during centrifugation.

Now specified:

"Take 10-20 µl of the filtered cell suspension and count the cells during centrifugation using a Bürker chamber."

9. Please include a one-line space between each protocol step and then then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive

story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The formatting of the revised manuscript was changed according to this comment

10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and should be in line with the Title of the manuscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

In the revised version of our manuscript, we highlighted in green steps that should be visualized in video.

4. Please modify the Result section to include all the observations and conclusions you can derive from the Figures. The Results section should focus on the effectiveness of your technique backed up with data.

In the revised version of our manuscript, we added new figures and created results section which now contains a detailed description of figures and representative results.

11. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

In revised manuscript we changed structure of figures significantly and added their detailed description. Each figure now has its title and description.

12. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

In revised manuscript we changed structure of figures significantly and added their detailed description. Each figure now has its title and description.

13. Figure 3: Please describe the x and y-axes.

In the revised version of our manuscript the original figure 3 has been replaced with a different figure.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods

e) Any future applications of the technique

We revised and completely rewrote the discussion. Now it precisely follows the guidelines.

15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references.

In the revised version of manuscript, we updated the references according to the official JoVE style.

*16. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an **xls/xlsx file**. Please sort the Materials Table alphabetically by the name of the material.*

We revised a table of materials which now contains all used materials. We submit this list in a separate .xlsx file.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript provides a simple and efficient protocol for the isolation of dental cells for downstream single-cell RNA seq analysis. The protocol can be virtually applied to any application or study based on single-dental-cells

Major Concerns:

I do not have major concerns. The manuscript is clear and easy to follow. The protocol is relevant for anybody working in the dental research field.

Minor Concerns:

- I suggest implementing schematic representations of the various steps in the already provided timeline. I know that the protocol will be accompanied by a video, but nevertheless, a graphical screenshot of the key passages could make the protocol even clearer

We are grateful for this comment. In revised version we added a schematic representation of the protocol which also contains temperatures and expected times for higher clarity.

- A table will all the materials and the solutions needed would be useful. The authors already provided a Table of materials, but it does not contain everything (e.g. scalpels, tweezers, industrial bench vise...). This would make the use of the protocol more efficient.

We revised the Table of materials which now contains all the necessary tools. Table containing the composition of used solutions has been provided as a supplementary table. We also specified the type of bench vise in the main text.

"NOTE: The composition of the utilized solutions is summarized in Supplementary table 1. "

"2.8.2 Place the tooth into three thick-walled sterile plastic bags and use a cast iron benchtop engineer's vise to crack the tooth. Use vise jaws with a flat surface to avoid penetrating the bags."

Reviewer #2:

Manuscript Summary:

This is an extremely useful protocol for researchers that use the tooth as a model system to dissect genetic and molecular gene networks.

We thank this reviewer for a very positive feedback to our manuscript.

Major Concerns:

None

Minor Concerns:

None

Reviewer #3:

This is an interesting study about the extraction of single-cell suspension from human and mouse dental pulp for single-cell sequencing. However, as a research focused on experimental method, some detailed points need to be added and modified for publication.

1.Compared with human dental pulp, mouse pulp tissue is extremely small, especially molars. In addition, cell loss is often caused in the process of FACS. Therefore, it is very necessary to state in the manuscript the approximate number of cells that can be obtained from each mouse (molar/incisor). And how many mice are needed in order to get enough cells for 10x genomics scRNA-seq (usually 20 000 cells).

Indeed, this is an important issue which deserves clarification. In the revised version of manuscript, we created new results section which now includes quantification of the exemplary experiment on mouse incisor. Unfortunately, due to the very short time for revisions (2 weeks) we were unable to perform this experiment on mouse molars and human teeth. However, since the greatest emphasis of the whole protocol is put on the incisor, we believe that this new result section serves as useful proof of concept also for other types of tissue.

Specifically, we added the following relevant parts of result:

"... To check the number of live/dead cells in the final suspension the PI (propidium iodide) staining was performed (Fig. 2C). The P3 population containing PI^- (living) cells was 98.4 % out of the parent P2 population and 35.5 % out of the total number of events. The total number of filtered out, dead (PI^+) cells was 1887.

The final number of obtained cells without viability staining suitable for RNA-seq (P2) counted 118 199 cells from two mouse incisor pulps. This means the number of almost 60 000 living cells from one mandibular incisor.

To clarify the number of immune cells in the final single-cell suspension two approaches were used. Firstly, the CD45 antibody staining and subsequent FACS analysis were used. As a complementary method, the total number of immune cells (CD45+) in scRNA-seq data was analysed. FACS analysis showed 14,44 % of CD45+ cells (13.20 % alive and 1.24 % dead)(Fig.3A). Analysis of scRNA-seq data showed 10,90 % of CD45-expressing cells (Fig.3B). The decrease of CD45+ cells in scRNA-seq data can be caused by additional thresholding during scRNA-seq analysis."

2.In addition, as for the molars of mice are very small, exposing the pulp is a very difficult task under naked eye. In the experiment, whether a stereo microscope was used for operation. Photos of the procedure for obtaining the pulp of mouse molars should be added.

We realize that our previous note positioned between 2.5 and 2.6: "NOTE: From this point forward work on ice. Further dissection of mouse incisors and molars are performed under a stereomicroscope with black background." might be overlooked. For this reason, we clarified and specified now using stereomicroscope in our protocol by modifying of 2.7.3 and adding new point 2.7.4:

"Once the pulp cavity is reached, carefully dissect dental pulp using pair of sharp tip tweezers and place the soft tissue of the dental pulp into a droplet of fresh ice-cold HBSS in the middle of a 10 cm Petri dish kept on ice. Since mouse molar pulps are extremely small, adapt magnification on the stereomicroscope accordingly."

Moreover, in revised version we added a schematic graphical representation of whole protocol. We feel that photo documentation might be redundant since whole protocol will be professionally filmed.

3.Regarding mouse and human dental pulp, what is the approximate ratio of immune cells? If the proportion of immune cells is too high, is CD45 staining required to exclude immune cells in FACS?

Immune cells were intentionally kept in the analysis. Our previous results show that immune system might play an important role in maintaining tissue homeostasis (Krivanek et al., 2020; and unpublished data). To address the question about the ratio of immune cells out of all cells

we provide two types of quantifications in the revised version of our manuscript. Firstly, we quantified number of immune cells using CD45 antibody and FACS and then we compared this number with the amount of CD45^{+} immune cells from the scRNA-seq analysis. Data are shown now in the Fig. 3 and discussed in results section as following:

„To clarify the number of immune cells in the final single-cell suspension two approaches were used. Firstly, the CD45 antibody staining and subsequent FACS analysis were used. As a complementary method, the total number of immune cells (CD45+) in scRNA-seq data was analyzed. FACS analysis showed 14,44 % of CD45+ cells (13.20 % alive and 1.24 % dead) (Fig.3A). Analysis of scRNA-seq data showed 10,90 % of CD45-expressing cells (Fig.3B). The decrease of CD45+ cells in scRNA-seq data can be caused by additional thresholding during scRNA-seq analysis."

4.In the preparation of mouse dental pulp single cell suspension, whether the whole tooth is cut and digested together or the dental pulp tissue is digested separately after the dental pulp tissue is extracted.

We are grateful for this comment. Now we added more detailed description for manipulation with both mouse incisor and molar tissue as following:

2.6.4. *"Place dissected soft tissue into a droplet of fresh ice-cold HBSS in the middle of a 10 cm Petri dish. Keep on ice"*

and:

2.7.4. *"Once the pulp cavity is reached, carefully dissect dental pulp using pair of sharp tip tweezers and place the soft tissue of the dental pulp into a droplet of fresh ice-cold HBSS in the middle of a 10 cm Petri dish kept on ice. Since mouse molar pulps are extremely small, adapt magnification on the stereomicroscope accordingly.*

5.In the entire manuscript, only three pictures are provided, and none of them can reflect the most important or complicated steps in obtaining human or mouse pulp tissue. More detailed pictures or videos of experimental operations are suggested to be provided.

We agree. In revised version we added a schematic graphical representation of whole protocol and a figure with representative results (Fig. 1). This will help to follow the protocol much easier. Moreover, whole protocol will be professionally filmed and commented after manuscript is accepted according to standard JoVE guidelines: https://www.jove.com/authors/publication.

Reviewer #4:

Manuscript Summary:

This manuscript describes a simple method isolate single cells from ECM-rich tissues for transcriptomic purposes such as single cell RNAseq. The main attraction of this protocol lies in its brevity and simplicity: cell incubation at temperatures higher than 4C are limited to 15-20 minutes to minimize gene induction in response to cell stress. The overall description is appropriate to follow the procedure. However, some validation steps could be included (see below) to allow for troubleshooting if necessary.

Major Concerns:

A critical point of any isolation procedure is the number and representation of cells isolated. Can the authors point out how recovery compares to other isolation procedures (recovery rate)? Also, do the authors have data on relative representation of cell populations following a 'standard' isolation versus the isolation method presented in this manuscript? As a user, this would be a critical component. I realize that this is a method paper, so documentation does not need to be exhaustive, but should at least be supportive.

In the revised version of manuscript, we added a result section with a detailed description of FACS analysis and, importantly, with the quantifications of live/dead cells. This also includes the quantification of cell yield on the example of mouse incisor. Finally, we analyzed the ratio of immune cells within the cell suspension by FACS analysis and in the final scRNA-seq data. These data prove the efficiency of presented protocol. Comparison of different protocols was unfortunately beyond the scope of this manuscript and was not possible to be performed within a short period given to revise the manuscript (14 days).

Figure legends are missing. While Fig. 1 is largely self-explanatory, Figs. 2 & 3 are not. Please describe what you show.

In the revised version of the manuscript, we changed the structure of figures. We added a schematic representation of the protocol which also contains temperatures and expected times (from previous Figure 1) for higher clarity. Figure 2 now represents updated sorting strategy which emphasize the numbers of different gates and, importantly numbers of dead cells. Figure 3 was updated and better described. Further we added a figure where we quantified amount of immune cells using CD45 antibody by FACS and then the comparison with the amount of CD45$^+$ (Ptprc$^+$) immune cells from the scRNA-seq analysis was performed.

The figure legends were added:

***"Figure 1. Schematic representation of protocol.** Different steps, including temperature conditions and expected time, for preparation of single-cell suspension from mouse and human teeth are represented.*

***Figure 2. Example of gating strategy.** FSC-A and SSC-A gating was used to produce the P1 gate which reflects cell population with expected size and granularity and filters out the cell and extracellular matrix debris and the majority of oversized events (A). Subsequently, the P1 population was plotted in FSC-H and FCS-A plot which enabled to filter out cell doublets (B). This P2 population was then analyzed for the presence of dead cells by propidium iodide (C). Numbers of events/cells per each gate are represented in D. (FSC-A – forward scatter, area; SSC-A – side scatter, area; FSC-H – forward scatter, height; PI – propidium iodide)*

***Figure 3. Quantification of immune cells.** Quantification of immune cells was performed by FACS analysis of cells stained with anti-CD45 antibody together with Live/Dead analysis using propidium iodide staining (A). Further quantification of immune cells was performed during scRNA-seq analysis (B). (CD45-APC – anti-CD45 allophycocyanin conjugated antibody; PI – propidium iodide; t-SNE – t-distributed stochastic neighbor embedding"*

Minor Concerns:

Isoflurane anesthesia/euthanasia. The open drop method is not approved anymore by all animal use and care committees. Maybe you want to provide an alternative (preferred), such as the use of an anesthetic machine or pentobarbital (Euthanyl) overdose via i.p. injection.

Thank you for this valuable comment. Regulations for euthanizing of experimental animals indeed significantly vary depending on local regulations. Based on this we changed this paragraph as following:

1.1 "Prepare the experimental animals (mouse) and euthanize them according to the local regulations.

CAUTION: Regulations for humane euthanizing of experimental animals varies locally. Always follow valid local regulations.

'Sacrifice' has a spiritual connotation, better use 'euthanize'.

Now we fixed this issue and substituted term "sacrifice" by "euthanize" in the whole manuscript.

Reviewer #5:

Manuscript Summary:

A critical step in scRNA sequencing of complex tissues is the obtention of single cell suspensions of good quality. This manuscript describes a protocol for obtaining such suspension from mouse and human teeth (incisors and molars), but could be generalized for other mineralized tissues. The protocol is surely of interest and provided with great details, which is really good. However, the efficacy of the protocol remains to be demonstrated (see below: sections are missing), and quantatitative results are needed to guide readers in handling the protocol.

Major Concerns:

There is no result section and the figure legends are also missing: is that really wanted or did something go wrong in submitting the article/compiling the pdf? To make this protocol fully useful to readers, we need some quantitative results, which will allow the reader to evaluate if he performs well with the protocol. For example, how much cells were obtained for each type of tooth? What were the respective proportions of debris, dead cells and viable cells? Later after sequencing, how were the quality controls for the different type of experiments (e.g. give medians, on top of showing fig3. Precise if the experiment shown in Fig3 results from cell sorting or direct sequencing of the cell suspension, if possible show different type of results, with and without facs cell sorting).

In the revised version of manuscript, we added a result section with a detailed description of FACS analysis and, importantly, with the quantifications of debris, doublets and live/dead cells. This also includes the quantification of cell yield on the example of mouse incisor as described in results:

"The prepared single-cell suspension was analyzed and sorted using FACS (Fig. 2). Firstly, the FSC-A (forward scatter, area) and SSC-A (side scatter, area) plotting was applied, and an appropriate gating strategy was used to select a population with expected size and granularity

to filter our cell debris and cell doublets or aggregates (Fig 2A). This selected population (P1) counting 38 % of all events was further used and FSC-A and FSC-H (forward scatter, height) parameters were applied to remove the remaining cell doublets (Fig. 2B). The population without cell doublets (P2) counting 95 % of P1 can be subsequently used for scRNA-seq. Alternatively, additional gating can be used to select the population of interest (e.g., expression of fluorescent proteins or live/dead staining). To check the number of live/dead cells in the final suspension the PI (propidium iodide) staining was performed (Fig. 2C). The P3 population containing PI^- (living) cells was 98.4 % out of the parent P2 population and 35.5 % out of the total number of events. The total number of filtered out, dead (PI^+) cells was 1887."

Previous figure 3 was removed from the revised manuscript since it was previously described in our recent paper (Krivanek et al., 2020, Supplementary Figure 1b,c) and was not relevant to the remaining parts of the manuscript which are focused purely on the isolation protocol and not the scRNA-seq itself. This figure was substituted by the quantification of immune cells using CD45 antibody by FACS and then the comparison with the amount of $CD45^+$ immune cells from the scRNA-seq analysis was performed:

"To clarify the number of immune cells in the final single-cell suspension two approaches were used. Firstly, the CD45 antibody staining and subsequent FACS analysis were used. As a complementary method, the total number of immune cells (CD45+) in scRNA-seq data was analyzed. FACS analysis showed 14,44 % of CD45+ cells (13.20 % alive and 1.24 % dead) (Fig.3A). Analysis of scRNA-seq data showed 10,90 % of CD45-expressing cells (Fig.3B). The decrease of CD45+ cells in scRNA-seq data can be caused by additional thresholding during scRNA-seq analysis."

These data prove the efficiency of presented protocol. All our previous scRNA-seq experiments were performed after FACS, thus, unfortunately we are unable to compare these results with data without FACS sorting. Comparison of different protocols was unfortunately beyond the scope of this manuscript and was not possible to be performed within a short period given to revise the manuscript (14 days).

The authors made a number of choices based on litterature (e.g. keep cells at 4°C), but they do not show that these choices indeed give better results. If they did not perform comparisons of different experimental settings, they could at least show that stress genes are poorly activated with their protocol, compare different measures of scRNAseq quality with the range obtained for other complex tissues.

We understand the raised concerns. Indeed, many our choices were based on the previous findings. As an example, the temperature during the procedure and overall time needed to isolate single cells have been shown to be critical. In our protocol we utilized this knowledge and minimized these critical factors. We carefully considered the possibility of comparing scRNA-seq data obtained using our protocol to other datasets obtained from different complex tissues utilizing different protocols. However, the comparison will not provide any representative results as these datasets differ in several key variables (type of tissue, source of the tissue, isolation protocol, scRNA-seq platform or handling differences). Therefore, these datasets are incomparable.

In the revised manuscript we added more relevant references which focus to the isolations of single cells out of various tissues:

„ These usually include trypsin, collagenases, dispases, papain [1–4] or other, commercially available enzyme mixtures like Accutase, Tryple etc. [5]. The most critical part, affecting the

transcriptome quality, is the enzymatic digestion. It was shown that prolonged incubation with enzymes at 37 °C influences the gene expression and causes the upregulation of many stress-related genes [6–9]. The other critical parameter of the isolation process is its overall length as it has been shown that cell transcriptomes undergo changes after the death of the individual [10]. Taken these two together, we developed an efficient protocol for gentle isolation of single cells from murine and human teeth which is faster than other, previously utilized protocols for isolation of cells from complex tissues [1, 4, 5, 7, 9, 11, 12].”

Serum was added to washing medium. Can the authors discuss this choice? Is this based on litterature evidence that serum improves results and does not induce a transcriptional response in cells?

In revised version of manuscript, we added reasoning of using FBS as following:

“NOTE: Collagenase P is activated by Calcium, whose amount is already sufficient in the lyophilized powder. Therefore, it is unnecessary to enrich the enzyme mix with calcium. Collagenase P is not inactivated by FBS but can be inactivated by chelating agents (e.g., Ethylenediaminetetraacetic - EDTA). Stopping the dissociation process is ensured by diluting the Collagenase P in Wash solution (2% FBS in HBSS). It has been shown that adding FBS increases cell viability and also the final number of cells after sorting[13].”

Minor Concerns:

Weird sentence:
For this purpose, an endogenously labelled cells are much more suitable

This sentence was removed in the updated manuscript.

References

1. Debnath, S. et al. Discovery of a periosteal stem cell mediating intramembranous bone formation. Nature. **562** (7725), 133–139, doi: 10.1038/s41586-018-0554-8 (2018).

2. Kanton, S., Treutlein, B., Camp, J.G. Chapter 10 - Single-cell genomic analysis of human cerebral organoids. Methods in Cell Biology. **159**, 229–256, doi: 10.1016/bs.mcb.2020.03.013 (2020).

3. Price, F.D. et al. Inhibition of JAK-STAT signaling stimulates adult satellite cell function. Nature Medicine. **20** (10), 1174–1181, doi: 10.1038/nm.3655 (2014).

4. van den Brink, S.C. et al. Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations. Nature Methods. **14** (10), 935–936, doi: 10.1038/nmeth.4437 (2017).

5. Chiba, Y. et al. Single-Cell RNA-Sequencing From Mouse Incisor Reveals Dental Epithelial Cell-Type Specific Genes. Frontiers in Cell and Developmental Biology. **8**, doi: 10.3389/fcell.2020.00841 (2020).

6. O'Flanagan, C.H. et al. Dissociation of solid tumor tissues with cold active protease for single-cell RNA-seq minimizes conserved collagenase-associated stress responses. Genome Biology. **20** (1), 210, doi: 10.1186/s13059-019-1830-0 (2019).

7. van Velthoven, C.T.J., de Morree, A., Egner, I.M., Brett, J.O., Rando, T.A. Transcriptional Profiling of Quiescent Muscle Stem Cells In Vivo. Cell Reports. **21** (7), 1994–2004, doi: 10.1016/j.celrep.2017.10.037 (2017).

8. Miyawaki-Kuwakado, A. et al. Transcriptome analysis of gene expression changes upon enzymatic dissociation in skeletal myoblasts. Genes to Cells. **26** (7), 530–540, doi: 10.1111/gtc.12870 (2021).

9. Mattei, D. et al. Enzymatic Dissociation Induces Transcriptional and Proteotype Bias in Brain Cell Populations. International Journal of Molecular Sciences. **21** (21), 7944, doi: 10.3390/ijms21217944 (2020).

10. Ferreira, P.G. et al. The effects of death and post-mortem cold ischemia on human tissue transcriptomes. Nature Communications. **9** (1), 490, doi: 10.1038/s41467-017-02772-x (2018).

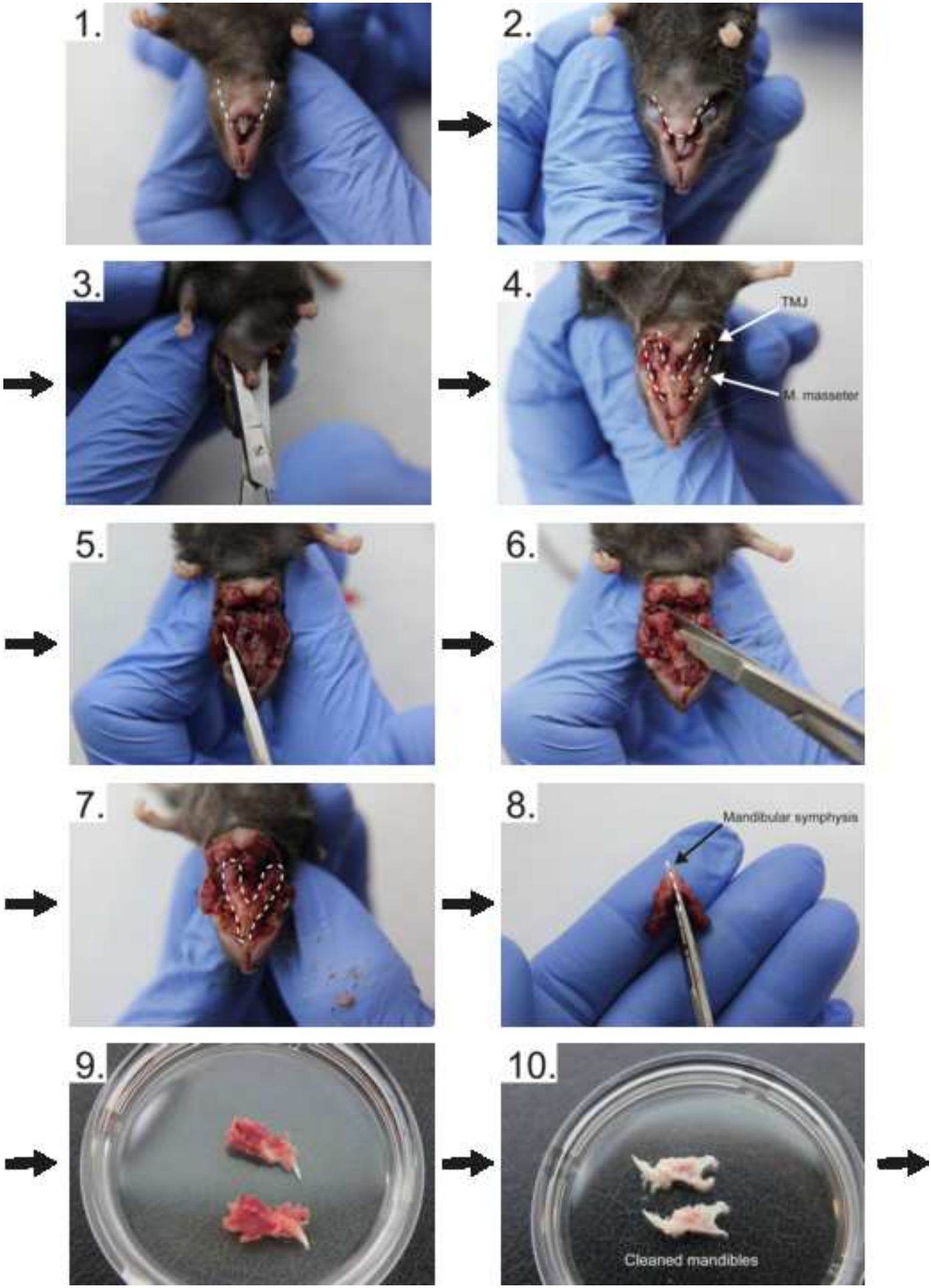
11. He, W., Ye, J., Xu, H., Lin, Y., Zheng, Y. Differential expression of α6 and β1 integrins reveals epidermal heterogeneity at single-cell resolution. Journal of Cellular Biochemistry. **121** (3), 2664–2676, doi: 10.1002/jcb.29487 (2020).

12. Machado, L. et al. In Situ Fixation Redefines Quiescence and Early Activation of Skeletal Muscle Stem Cells. Cell Reports. **21** (7), 1982–1993, doi: 10.1016/j.celrep.2017.10.080 (2017).

13. Song, X. et al. Improved Strategy for Jet-in-air Cell Sorting With High Purity, Yield, Viability and Genome Stability. doi: 10.21203/rs.3.rs-127241/v1 (2021).

Supplementary Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary figure 1.png



Supplemental table 1

Wash solution – 50 ml

Component	Volume	Manufacturer	Cat. number
FBS	1 ml	Biosera	FB-1101/500
HBSS	49 ml	SIGMA	H6648

Digestion solution – 2.5 ml

Component	Volume/amount	Manufacturer	Cat. number
Collagenase P	7.5 U	Roche	11213857001
HBSS	2.5 ml	SIGMA	H6648

Storing solution – 10 ml

Component	Volume/weight	Manufacturer	Cat. number
BSA	4 mg	Roche	10735078001
HBSS	10 ml	SIGMA	H6648