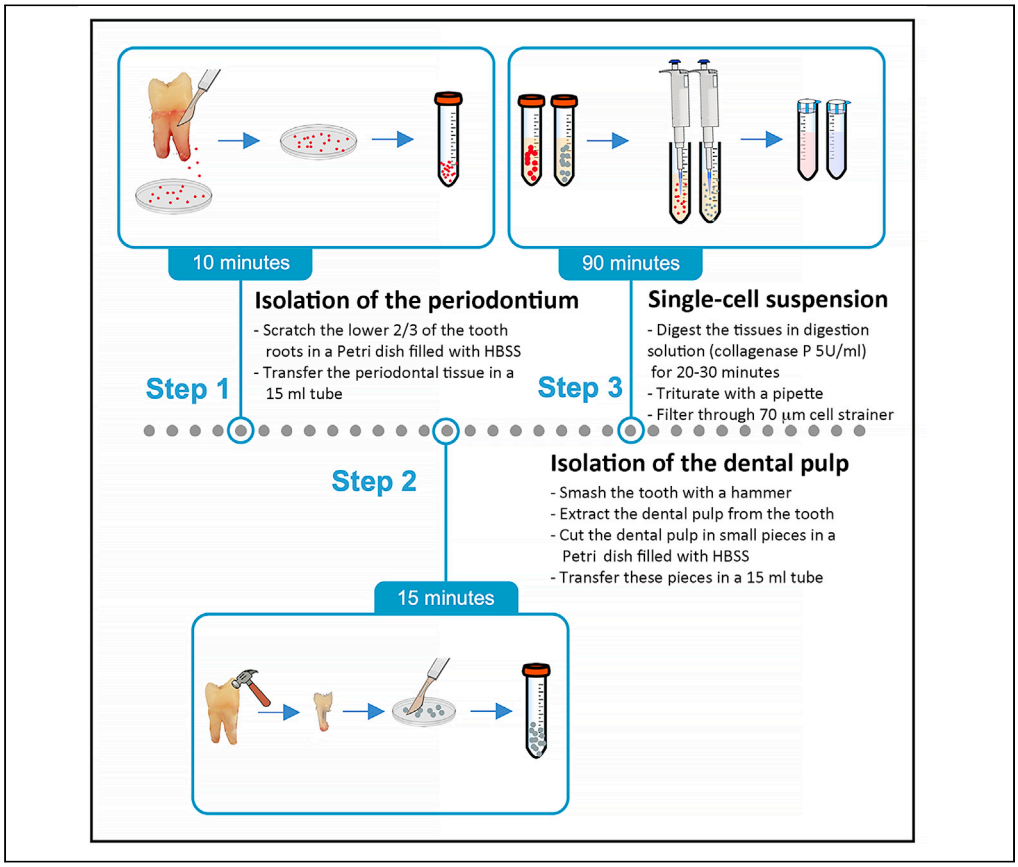
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Isolation of dental pulp and periodontal cells from human teeth for single-cell RNA sequencing



Teeth and the surrounding periodontal tissues are affected by many pathologies that compromise their integrity and significantly affect life quality. The study of the main dental tissues, the dental pulp and periodontium, is made arduous by their close association with highly mineralized tissues (dentin, cementum, and alveolar bone). Here we describe a protocol to isolate all cells composing human dental pulp and periodontium for single-cell RNA sequencing analysis.

Pierfrancesco Pagella, Bernd Stadlinger, Thimios A. Mitsiadis

thimios.mitsiadis@zzm.uzh.ch

Highlights

Cell isolation from human teeth is challenging due to contact with hard tissues

Protocol to isolate all cell types from the dental pulp of human teeth

Protocol to isolate all cell populations from the periodontium of human teeth

Viability of cell populations compatible with single-cell RNA sequencing analysis

Pagella et al., STAR Protocols *2*, 100953
December 17, 2021
https://doi.org/10.1016/j.xpro.2021.100953

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Isolation of dental pulp and periodontal cells from human teeth for single-cell RNA sequencing

Pierfrancesco Pagella,[1,3,4] Bernd Stadlinger,[2] and Thimios A. Mitsiadis[1,5,*]

[1]Orofacial Development and Regeneration, Institute of Oral Biology, University of Zurich, Zurich, Switzerland

[2]Clinic of Cranio-Maxillofacial and Oral Surgery, Center of Dental Medicine, University of Zurich, Zurich, Switzerland

[3]Present address: Wallenberg Center for Molecular Medicine; Department of Biomedical and Clinical Sciences (BKV), Division of Molecular Medicine and Virology (MMV), Linköping University Linköping, Sweden

[4]Technical contact

[5]Lead contact

*Correspondence: thimios.mitsiadis@zzm.uzh.ch
https://doi.org/10.1016/j.xpro.2021.100953

SUMMARY

Teeth and the surrounding periodontal tissues are affected by many pathologies that compromise their integrity and significantly affect life quality. The study of the main dental tissues, the dental pulp and periodontium, is made arduous by their close association with highly mineralized tissues (dentin, cementum, and alveolar bone). Here we describe a protocol to isolate all cells composing human dental pulp and periodontium for single-cell RNA sequencing analysis.
For complete details on the use and execution of this protocol, please refer to Pagella et al. (2021).

BEFORE YOU BEGIN

This protocol describes the isolation of dental pulp and periodontal cells from healthy, freshly extracted human teeth. All Materials required are listed in the attached key resources table.

The protocol was successfully applied to different types of human teeth, mainly to third molars (wisdom teeth). We selected preferentially teeth extracted from patients between 18 and 35 years of age, as dental pulp size significantly decreases with age, due to the continuous (lifespan) deposition of secondary dentin by odontoblasts (Goldberg et al., 2011). Beware of the legal requirements for the access to human specimens applicable at your institution. Our study (Pagella et al., 2021) was performed on completely anonymized teeth; the procedure for the collection of anonymized human dental pulp and periodontal cells at the Center of Dental Medicine (ZZM) of the University of Zurich was approved by the Ethic Commission of the Kanton of Zurich (reference number 2012-0588) and the patients gave their written informed consent.

Pivotal for the success of the single-cell RNA sequencing procedure is to minimize the operation time between the tooth extraction and the dental pulp and periodontal tissues isolation procedure. Therefore, all solutions and instruments listed below, and in the annexed key resources table, should be prepared and ready to use before tooth extraction by the dentists.

Immediately after extraction, the teeth should be stored in sterile either NaCl 0.9% / Phosphate Buffer Saline (PBS) or Hank's Balanced Salt Solution (HBSS). Thereafter, the teeth should be transferred on ice from the clinic (operation room) to the research laboratory. 20 min or less is considered the ideal timing between the tooth extraction procedure and the beginning of the isolation of the dental pulp and periodontal tissues.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human teeth	Center of Dental Medicine, University of Zurich	N/A
Chemicals, peptides, and recombinant proteins		
Trypsin (2.5%), no phenol red	Gibco	Cat. N° 15090046
Other		
Falcon® 70µm Cell Strainer (or equivalent-size strainer)	Falcon	Cat. N° FAL352350
Hank's Balanced Salt Solution HBSS (10X), no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	Cat. N° 14185045
Fetal Bovine Serum – heat inactivated (or equivalent serum)	Sigma-Aldrich	Cat. N° F0804-500ML
Albumin Fraction V, biotin-free min. 98 %, for molecular biology	Carl Roth	Cat. N° 0163.2
Trypan blue 0.4% solution (or equivalent)	Sigma-Aldrich	Cat. N° T8154
Corning ® Syringe filter 0.22 µm (or equivalent-size filter)	Corning	Cat. N° CLS431219-50EA
Spring scissors - 8mm cutting edge (or equivalent)	Fine Science Tools	Cat. N° 15009-08
Dumont#7 curved forceps (or equivalent)	Fine Science Tools	Cat. N° 11271-30
Dumont #5 forceps (or equivalent)	Fine Science Tools	Cat. N° 11255-20
Scalpel Handle #4 (or equivalent)	Fine Science Tools	Cat. N° 10004-13
Scalpel Blades #20 (or equivalent)	Fine Science Tools	Cat. N° 10020-00
151 Cryer Forceps (or any equivalent forceps that allow tooth hold)	Hu-Friedy	Cat. N° F151
Leica M80 Stereomicroscope (or equivalent)	Leica Microsystems	N/A
Neubauer Haemocytometry chamber (or equivalent)	Electron Microscopy Sciences	Cat. N° #68052-14, 68052-15

MATERIALS AND EQUIPMENT

Buffers and solutions

Timing: 15 min

Resuspend Collagenase-P at 20 U/mL in sterile HBSS; aliquot (2 mL/aliquot) and store at −20°C.

For each tooth preparation, dilute 2 aliquots of Collagenase-P (20 U/mL) in 3 volumes of HBSS to obtain 16 mL of a Collagenase-P 5 U/mL solution.

Warm up the Collagenase-P 5 U/mL solution to 37°C. This will take approximately 10 min.

Prepare 50 mL of HBSS + 0.02% bovine serum albumin. Filter through a 0.22 µm filter and store at 4°C up to 1 month.

Prepare 50 mL of HBSS + 2% foetal bovine serum (FBS). Filter through a 0.22 µm filter and store at 4°C up to 1 month.

Sterilization of instruments

Timing: 1–3 h (depending on autoclave cycle)

Autoclave 2 sets of dissections forceps, 1 dissection scissor.

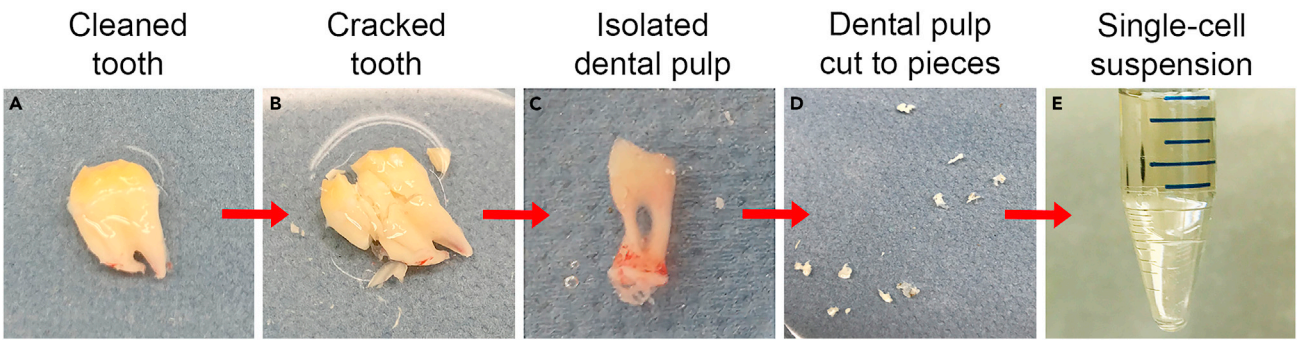


Figure 1. Overview of dental pulp isolation and digestion to single-cell suspension

(A) After removal of the periodontium by scratching the lower two-thirds of the tooth root, wipe the tooth with 70% ethanol.

(B) Crack the tooth with a hammer or a press with a single, firm stroke.

(C and D) (C) Isolate the dental pulp, (D) cut it into small pieces (approx. 2 mm of diameter) and transfer it into a 15 mL tube containing the digestion solution.

(E) By the end of the digestion, the cell suspension should be devoid of clumps and tissue fragments.

STEP-BY-STEP METHOD DETAILS

Isolation of periodontium

Timing: approx. 10 min

1. Fill one 35 mm Petri dish with 2 mL of sterile, cold Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific, Reinach, Switzerland). Keep it on ice.
2. Rinse the tooth in sterile, cold HBSS
3. Hold the tooth crown with a tooth holder.
4. With a surgical blade, scrape the periodontium into the Petri dish filled with sterile HBSS. Limit scraping to the apical two-thirds of the root, to minimize contamination from the gingival region. Troubleshootings 1 and 2
5. Collect with a P1000 pipette all the HBSS and the periodontium fragments in the Petri dish.
6. Transfer the HBSS with the periodontium fragments in a clean 15 mL Falcon Tube.
7. Store the periodontium suspension on ice until completion of dental pulp isolation (ideally, not longer than 1 h).

Isolation of the dental pulp

Timing: approx. 15 min

In this step, the tooth will be cracked, and the dental pulp extracted. An overview of the procedure for the dental pulp extraction is provided in Figure 1.

8. Carefully wipe with 70% ethanol the surface of the tooth crown and of the roots to remove eventual remnants of the periodontium and gingival tissues.
9. Carefully remove the ethanol from the surface.
10. Dip the tooth in sterile HBSS.
11. Fill one 35 mm Petri dish with cold, sterile HBSS.
12. Crack the tooth with a press or a hammer. Apply a single, powerful stroke to crack enamel and dentin, without generating small debris and applying excessive and prolonged pressure on the dental pulp. A single impulse generally does not cause smashing/squeezing of the pulp, but it rather cracks only the surrounding dentin and enamel.
13. With a first set of curved forceps, carefully separate the cracked fragments of the tooth. The dental pulp will be exposed.

14. With a second, sterile set of straight forceps, remove the dental pulp from the pulp chamber.
15. Place the dental pulp in the Petri dish, previously filled with HBSS.
16. Place the isolated dental pulp under a stereomicroscope, and with the second set of forceps remove any remnants of hard tissues.
17. With a scalpel or with fine dissection scissors, chop the dental pulp in small pieces.
18. Collect with a P1000 pipette all the HBSS and the dental pulp fragments in the Petri dish and transfer them in a clean 15 mL Falcon Tube. Troubleshooting 3.
19. Store the dental pulp suspension on ice for up to 30 min.

Note: Longer times have not been tested.

Dissociation of dental pulp and periodontium to single-cell suspension

Timing: approx. 90 min

⚠ CRITICAL: perform all the following steps on ice, whenever possible.

20. Centrifuge the dental pulp and the periodontium, now each in approximately 5 mL HBSS in a 15 mL falcon tube, at 300×*g* for 10 min at 4°C.
21. Carefully decant the supernatant. Resuspend the dental pulp and the periodontium each in 8 mL of prewarmed Collagenase-P 5 U/mL.
22. Close the falcon tubes and seal them with parafilm.
23. Place the falcon tubes into a shaker.
24. Incubate the tissues at 37°C, shaking horizontally (120–160 rpm), for 40 min.
25. Every 10 min, triturate the tissues by pipetting up and down with a p1000 with slightly cut tip, then place back to digest.
26. At the end of the incubation time, take out the falcon tubes and place them on ice.
27. Disaggregate the remaining pieces of tissue by pipetting up and down with a p1000 first with cut tip, then with normal tip.

Note: Some filaments might remain (e.g., nerve fibres). Moreover, in case of incomplete removal of mineral fragments in step 11, these will always be visible in the cell suspension. Troubleshooting 4 and Troubleshooting 5

28. Fill up the falcon tubes with cold HBSS + 2% FBS. Centrifuge at 300×*g* for 10 min at 4°C.
29. Carefully decant the supernatant. Resuspend in 1 mL HBSS + 0.02% BSA. Pipette again thoroughly with p1000 with cut tip, then with a p1000 with normal tip, then with a p200 with normal tip.
30. Filter the cell suspension through a 70 μm filter, place filtered cells on ice.

Note: filtering through a 40 μm filter induces additional stress to the cells; it is however safer for avoiding the loading of cell duplets during successive steps. Filtering through a 40 μm filter is suggested only if the cells are immediately (< 20 min) processed with 10x Genomics® Single Cell Protocols. In our experience, we kept cells for a maximum of 90 min before proceeding with 10X Genomics Single-Cell RNA sequencing.

31. Take 10 μL of cell suspension and mix them with 10 μL of trypan blue 0.4%. Count the cells in a Burker or Neubauer chamber.
    a. The concentration should be at least 1000 cells/μL for the 10x Genomics single cell RNA sequencing pipeline.
    b. If lower, centrifuge at 300×*g* for 10 min at 4°C, then resuspend in the appropriate volume.
32. Cells can be further processed for 10x Genomics Single-Cell RNA sequencing.

a. *Optional:* In particular in the periodontium, red blood cells could represent a significant fraction of the samples. To minimize the fraction of erythrocytes further processed for 10X Genomics and single-cell RNA sequencing, after step 24 cell suspensions can be processed as described in the following protocol from 10X Genomics: https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/demonstrated-protocol-isolation-of-leukocytes-bone-marrow-and-peripheral-blood-mononuclear-cells-for-single-cell-rna-sequencing

EXPECTED OUTCOMES

A healthy dental pulp (age 18–35) from a third molar will provide approx. 1–2 million cells; a periodontium will provide 50,000 cells. With this protocol, we successfully isolated and described mesenchymal stem cells, fibroblasts, endothelial cells, Schwann/glial cells, immune cells, odontoblasts, and epithelial cells, and managed to detect important cellular heterogeneity within each of these cellular subtypes. Classical dental pulp cells and periodontal cells isolation protocols often rely on enzymatic digestion via combinations of Trypsin, Dispase and Collagenase (Rodas-Junco and Villicaña, 2017; Yasui et al., 2017; Athanassiou-Papaefthymiou et al., 2015; Hilkens et al., 2013; Morsczeck et al., 2005; Gronthos et al., 2000; About et al., 2000). We opted for a digestion solution containing only Collagenase to minimize the potential impact of the dissociation protocol on possibly fragile cell types inhabiting the dental pulp and the periodontium.

Teeth from different patients displayed high variability, in particular concerning immune cells.

LIMITATIONS

The protocol provides a rapid and robust dissociation pipeline for human dental tissues. Some cell types, such as neutrophils, were underrepresented in our specimens, possibly due to their low resistance to our dissociation procedure (Teng et al., 2017). Similarly, only a small proportion of odontoblasts are detached from the dentin, where their odontoblastic processes reside. Often pulp detachment from the dentin leads to ruptures of the odontoblastic processes from their bodies, thus killing odontoblasts.

In some cases, high levels of cell death were also detected in the periodontium (up to 40%), due to the combination of harsh tooth extraction from the alveolar bone and the mechanical detachment (scratching) of periodontal cells from the tooth roots.

TROUBLESHOOTING

Problem 1

Potential contamination of gingival cells in the periodontium (step 4).

Potential solution

As indicated in the protocol, wash the tooth thoroughly in sterile HBSS before scratching the periodontium. If large pieces of gingiva are attached to the extracted tooth, remove them before proceeding with the scratching of the periodontium. Always scratch only the lower two thirds of the roots to isolate the periodontium.

Problem 2

High cell mortality in the periodontium; scraping the periodontium with the cutting edge of the scalpel, although efficient, might lead to high cell mortality (step 4).

Potential solution

Scrape the periodontium using the non-cutting edge of the scalpel.

Problem 3

Persistence of mineralized tissue in the dissociation reaction; fragments of dentin persist during the digestion and remain associated with clumps of tissue (step 18).

Potential solution

Exerting too much force during the "tooth cracking" step might create small dentin fragments, which remain embedded in the dental pulp and impair proper dissociation and disturb pipetting and filtering. To avoid this issue, crack the tooth with a single, decisive stroke – being it via the press (best method) or a hammer.

Problem 4

Incomplete dissociation: after the described digestion time, clumps of tissues are still visible and do not dissolve upon pipetting (step 27)

Potential solution

Chopping the tissues into the smallest possible fragments before digestion is fundamental to ensure proper collagenase P access to the tissues; having too large pieces often results in incomplete digestion. In case of incomplete digestion, add Trypsin to the dissociation mixture at a final concentration of 0.25% (from a 10X stock, such as Trypsin 2.5%, Gibco, 15090046). Carefully monitor the dissociation reaction every 10 min and pipette up and down with a p1000 pipette to help tissue disaggregation. Stop the reaction by diluting the cell suspension in HBSS + 2% FBS, as indicated above, as soon as the dissociation is completed. Excessive trypsin incubation (more than 45 min) can result in the loss of delicate cell types and loss of surface antigens (Tsuji et al., 2017).

Problem 5

High variability between samples: relative abundance of cell types highly variable between samples (step 27).

Potential solution

A certain extent of variability between samples is normal and represents biological variability between patients. Moreover, if patients are completely anonymized as in our studies, the experimenter can assess solely the health status of the tooth, and not the overall health condition of the donor, which could affect in particular immune cells present in dental tissues.

Experimental variability can be affected by collagenase P batches; we indeed observed important variability in the dissociation efficiency between different collagenase P batches. We suggest performing the dissociation of samples all with the same batch of collagenase P.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thimios Mitsiadis (thimios.mitsiadis@zzm.uzh.ch).

Materials availability

This study did not generate new unique reagents. For specific details on availability please refer to the key resources table.

Data and code availability

This protocol did not generate/analyze datasets or code.

ACKNOWLEDGMENTS

This work was financially supported by the University of Zurich and by the Swiss National Science Foundation (SNSF) (grant no: 310030_197782).

AUTHOR CONTRIBUTIONS

Conceptualization, P.P., T.A.M.; formal analysis, P.P., B.S., T.A.M.; investigation, P.P., T.A.M.; methodology, P.P., T.A.M.; validation, P.P., B.S., T.A.M.; visualization, P.P., T.A.M.; writing – initial draft, P.P., T.A.M.; writing – review & editing, P.P., B.S., T.A.M; project administration, T.A.M.; resources, T.A.M.; supervision, T.A.M.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

REFERENCES

About, I., Bottero, M.J., de Denato, P., Camps, J., Franquin, J.C., and Mitsiadis, T.A. (2000). Human dentin production in vitro. Exp. Cell Res. *258*, 33–41.

Athanassiou-Papaefthymiou, M., Papagerakis, P., and Papagerakis, S. (2015). Isolation and characterization of human adult epithelial stem cells from the periodontal ligament. J. Dent. Res. *94*, 1591–1600.

Goldberg, M., Kulkarni, A.B., Young, M., and Boskey, A. (2011). Dentin: structure, composition and mineralization. Front. Biosci. (Elite Ed.) *3*, 711–735.

Gronthos, S., Mankani, M., Brahim, J., Robey, P.G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc. Natl. Acad. Sci. U S A *97*, 13625–13630.

Hilkens, P., Gervois, P., Fanton, Y., Vanormelingen, J., Martens, W., Struys, T., Politis, C., Lambrichts, I., and Bronckaers, A. (2013). Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell Tissue Res. *353*, 65–78.

Morsczeck, C., Gotz, W., Schierholz, J., Zeilhofer, F., Kuhn, U., Mohl, C., Sippel, C., and Hoffmann, K.H. (2005). Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. Matrix Biol. *24*, 155–165.

Pagella, P., de Vargas Roditi, L., Stadlinger, B., Moor, A.E., and Mitsiadis, T.A. (2021). A single cell atlas of human teeth. iScience *24*, 102405.

Rodas-Junco, B.A., and Villicaña, C. (2017). Dental pulp stem cells: current advances in isolation, expansion and preservation. Tissue Eng. Regen. Med. *14*, 333–347.

Teng, Y., Luo, H.R., and Kambara, H. (2017). Heterogeneity of neutrophil spontaneous death. Am. J. Hematol. *92*, E156–E159.

Tsuji, K., Ojima, M., Otabe, K., Horie, M., Koga, H., Sekiya, I., and Muneta, T. (2017). Effects of different cell-detaching methods on the viability and cell surface antigen expression of synovial mesenchymal stem cells. Cell Transpl. *26*, 1089–1102.

Yasui, T., Mabuchi, Y., Morikawa, S., Onizawa, K., Akazawa, C., Nakagawa, T., Okano, H., and Matsuzaki, Y. (2017). Isolation of dental pulp stem cells with high osteogenic potential. Inflamm. Regen. *37*, 8.