Cover Page

Rodrigo A. Somoza, PhD

Skeletal Research Center

Case Center for Multimodal Evaluation of Engineered Cartilage

Department of Biology

Case Western Reserve University

10900 Euclid Avenue

Cleveland, OH, 44106

rodrigosomoza@case.edu

Jean F. Welter

Skeletal Research Center

Case Center for Multimodal Evaluation of Engineered Cartilage

Department of Biology

Case Western Reserve University

10900 Euclid Avenue

Cleveland, OH, 44106

jean.welter@case.edu

Isolation of chondrocytes from human cartilage and cultures in monolayer and 3D

2. Materials

1. Appropriate Personal Protective Equipment (PPE)
    a. Lab coat
    b. Gloves
    c. Eye protection
2. Biological safety cabinet
3. Cell culture incubator
4. Inverted microscope
5. Tabletop centrifuge with rotor for 50 ml tubes
6. Pipette-Aid
7. Sterile 50 ml, polypropylene tubes
8. Sterile plastic disposable serological pipettes 10 and 25 ml
9. Wide-orifice pipet tips
10. T175 flasks
11. 100 mm Petri culture dishes
12. 20 ml syringes with 18 gauge needles
13. Sterile cell strainer, 70 µm pore size
14. Filter unit with receiver 0.2 µm
15. Sterile forceps (2)
16. #10 sterile scalpel or #10 scalpel blades + blade holders

17. Ethyl alcohol: 95%, 190 proof
18. Dulbecco's Phosphate Buffered Saline (DPBS), without calcium and magnesium 1X
19. Hanks' balanced salt solution (Sigma Chemical Co) 1X
20. DMEM-LG: Dulbecco's modified Eagle's medium with 1g/l glucose,
21. DMEM-HG: Dulbecco's modified Eagle's medium with 4g/l glucose
22. Antibiotic / Antimycotic, 100X stock solution 10,000 U of penicillin G, 10 mg of streptomycin, and 2.5 μg of amphotericin B per ml in water
23. FBS: Fetal Bovine Serum
24. Sodium pyruvate: 100X stock solution 100mM in water
25. Nonessential amino acids: 100X (Invitrogen)
26. ITS+: Recombinant Human Insulin 1.0 mg/ml, Human Transferrin 0.5 mg/ml, Selenite 6.7 x 10^{-4} mg/ml, Linoleic Acid 0.5 mg/ml, Bovine Serum Albumen 100mg/ml (R&D systems)
27. Dexamethasone stock solution: 10^{-4} M in Ethanol
28. Ascorbic acid--2 phosphate stock solution: 800 mM in water (Wako Chemicals)
29. Transforming growth factor β—1: 20 μg/mL in sterile 4 mM HCl containing 1 mg/mL bovine serum albumin
30. Complete chondrocyte medium: DMEM-HG, 1% antibiotic–antimycotic solution, 1% sodium pyruvate, 1% nonessential amino acids (Invitrogen), 1% ITS+, 10^{-7} M dexamethasone, 80 mM ascorbic acid-2 phosphate, transforming growth factor β-1 (10 ng/mL)
31. Serum supplemented DMEM: DMEM-LG with 10% FBS
32. Trypsin: 2.0 mg/ml in PBS
33. Testicular hyaluronidase: 2.0 mg/ml (330 Units/ml) in DMEM (Sigma Chemical), prepare fresh
34. Type II collagenase: 2.0 mg/ml (250 Units/ml) in DMEM (Worthington Biochemical Corporation), prepare fresh
35. Trypsin--EDTA: 0.05% trypsin in 0.53 mM EDTA
36. Milli-Q water (18.2 MΩ·cm)

37. Trypan Blue: 0.4% in PBS
38. Polypropylene v-bottom 96-well plates and lids

3. Methods

3.1 Chondrocyte isolation

1. General Safety: All human tissues should be considered to be potentially contaminated with bloodborne pathogens and so should be handled using Standard Precautions, including proper hand hygiene, use of PPE (gloves, lab coat, face shield), and proper disposal of waste, including liquids and sharps used in the processing of the tissue.
2. The fresh harvested cartilage sample is immediately placed into a sterile 50 ml centrifuge tube filled with DMEM-LG, supplemented with 1% antibiotic/antimycotic solution by the Operating Room team. The cartilage is kept at 4°C and then transported to the lab as expediently as possible, in these tubes, in a styrofoam box. Isolation steps should be performed within 48 hours.
3. Fresh tissue from a tissue procurement service should always be maintained in a saline solution and kept at 4°C during transport and storage. In our experience we have been able to recover viable chondrocytes even from samples that have been stored for 60 days before harvesting. Although long storage of cartilage tissue is not ideal for chondrocyte isolation, it provides flexibility that sometimes is needed to process this samples.
4. When planning the isolation, be aware that digestion protocols include an overnight incubation.

3.2 Isolation of chondrocytes with sequential digestion (recommended for articular cartilage):

1. Prepare complete chondrocyte medium
2. Prepare Enzyme Solutions for sequential treatment. All enzymatic digestion solutions should be sterile filtered (0.2 µm) prior to use:
    a. Trypsin

b. Testicular hyaluronidase (freshly prepared)

c. Type II collagenase (freshly prepared)

d. Trypsin (0.05%) and EDTA

3. If applicable, remove perichondrium or any repair tissue.
4. Cut 1 mm thick full-thickness slices of cartilage down to the bone using sharp dissection (*see* **Notes 1** and **2**)
5. Cut the cartilage slices into pieces approximately 1 mm^3.
6. Transfer the pieces into a sterile 100 ml glass flask with a stir-bar and flood with 20 ml enzymatic digestion solution (same volume for all enzyme solutions).
7. Place on a stirrer and digest at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% air using the following enzymatic sequence:

    a. Trypsin (30 minutes),

    b. Testicular hyaluronidase (60 minutes)

    c. Collagenase type II (overnight) (*see* **Note 3**).
8. Alternatively, pieces can be placed on a 100 mm Petri culture dish with 10 ml enzymatic digestion solution and placed on an orbital shaker.
9. Remove supernatant and add 10 ml FBS to it to stop, save this.
10. Digest the remaining undigested cartilage with 0.25% collagenase type II and 0.5% trypsin for 2-3 hours at humidified atmosphere. (*see* **Note 4**)
11. Remove the supernatant and stop the digestion with 10 ml FBS.
12. Either pool the supernatants or process separately, as preferred (*see* **Note 5**).
13. Strain through the sterile 70 μm pore size cell strainer to remove debris.
14. Centrifuge at 260 ×g for 10 minutes
15. Wash the pellet twice with DMEM-LG supplemented with 10% FBS (*see* **Note 6**)
16. Resuspend in complete chondrocyte medium

17. Determine cell number and viability using a hemacytometer and Trypan Blue vital dye. Plate at 200,000 cells per 10 cm Petri culture dishes in complete chondrocyte medium (*see* **Note 7**)
18. Incubate cells in cell-culture incubator at 37°C and 5%CO_2

3.3 Propagation and Routine Maintenance of Chondrocytes in 2D

1. Examine cultures under inverted microscope daily, note cell growth or any signs of contamination.
2. During the first week, individual cell colonies should be observed.
3. Change the medium every third or fourth day by aspirating and discarding spent medium, and replace with the 25 ml of fresh complete pre-warmed chondrocyte medium per flask.
4. Recap the flask(s), and return to the incubator.
5. Dispose of spent culture medium into 10% bleach.
6. Subculture is carried out when culture flasks are 80 -- 90% confluent. Primary cultures usually take 10 to 16 days before they are ready to be subcultured. If cells are ready to passage, trypsinize cells according to the trypsinization protocol, below, then:
7. Resuspend the cell pellet(s) in chondrocyte medium. For each T175 flask, resuspend cells in 3 ml of complete chondrocyte medium.
8. Thoroughly mix the suspension by pipetting up and down.
9. Remove an aliquot (20 µl) and dilute 1:1 with 0.4% Trypan Blue. Count cells and calculate cell density and the total number of cells using a hemacytometer.
10. Adjust cell concentration to 1×10^6 cells/ml.
11. Add 1 ml of the cell suspension to each T175
12. Add 25 ml complete chondrocyte medium
13. Cap the flask(s) and return to the incubator.

3.4 Trypsinization of monolayer-cultured chondrocytes

The purpose of this procedure is to remove the chondrocytes from the vessel surface for use, propagation, or cryopreservation. As with the other protocols in this chapter, Standard Precautions apply to any human-derived cultures. All applicable PPE and engineering controls should be used.

The attachment of cells to each other and to the culture substrate is mediated by cell surface glycoproteins and Ca^{2+} and Mg^{2+} ions. Proteins derived from serum also facilitate cell adhesion. To detach the cells from flask, remove culture medium, wash with DPBS and expose to Trypsin-EDTA.

1. Change the medium on the day before trypsinization
2. Thaw a sufficient number of 50 ml sterile aliquot of Trypsin--EDTA in a water bath at 37 ºC. Discard unused portion at the end of the procedure.
3. Prepare growth chondrocyte medium or freezing medium as needed.
4. Warm DPBS or Hanks' balanced salt solution
5. If there are a large number of flasks to trypsinize, do only 5 flasks at a time, as leaving the Trypsin--EDTA on the cells for more than 10 minutes decreases viability
6. For a T175 flask, add 20 ml of the pre-warmed DPBS to the side of the flask opposite the cells (to avoid dislodging the cells);
7. Rinse the cell layer by gently swirling the DPBS on cell layer
8. Discard rinse.
9. Add 8 ml of the pre-warmed Trypsin--EDTA to the side of the flask opposite the cells. Turn flask over to cover the cell layer completely. Incubate cells at 37 ºC in incubator for 5 minutes.
10. Check the progress of detachment by examining flasks under the inverted microscope to ensure that the cells have completely detached prior to recovery of the cells. Do not force the cells to detach before they are ready to do so, or clumping may result (*see* **Note 8**).
11. Add 8 ml of complete chondrocyte medium to each flask to stop the action of the trypsin. Disperse cells by repeated pipetting or gentle agitation.

12. Transfer the suspension(s) to sterile 50 ml conical centrifuge tube(s). Rinse flask with 10 ml of DPBS and add this to the suspension.
13. Centrifuge at 180 × g for 5 minutes at 37 ºC.
14. Aspirate the supernatant and resuspend the cell pellet in 5 ml of chondrocyte medium.
15. If multiple tubes are used, pool cell suspension in one tube. Count cells and then use, replate at 1×10^6 cells per T175 as needed.

3.5 Chondrocyte culture in 3D aggregate culture:

1. For use in aggregate culture, resuspend the trypsinized cells to a density 0.25×10^6 cells/mL in complete chondrocyte medium
2. Pipet a 200 µl aliquot into each well of a sterile polypropylene v--bottom 96 well plate for 0.25×10^6 cells per well (*see* **Notes 9** and **10**).
3. Centrifuge the plates at 500 × g for 5 minutes and then gently transfer to the incubator.
4. Aggregates will form within the first 24 hours.
5. After 24 hours, gently pipet the medium in the wells up and down a few times to detach the aggregates from the bottom of the wells, taking care not to disrupt the aggregates.
6. Change the medium per experimental protocol, but no less than every 3 days. Care should be taken not to aspirate the aggregates when changing the medium (*see* **Note 11**).
7. Aggregates can be harvested after 3 weeks, at this stage chondrocytes inside lacunae will be observed within the 3D cartilage ECM.
8. To harvest the aggregates, a wide-orifice pipet tip will usually allow the aggregates to be picked up and transferred for upstream analyses.

4. Notes

1:

Articular cartilage (especially in the knee) has different weight-bearing zones, which have adapted to different physiological loading conditions, and that result in different tissue and chondrocyte properties. Therefore, isolating chondrocytes specifically from different weight-bearing areas may be of research interest. The same isolation protocol can be used for either zone (weigh--bearing or non-weight bearing).

2:

When cutting slices of cartilage, it is important not to harvest tissue from the bone-cartilage interphase. The presence of vascular channels in subchondral bone may contaminate chondrocyte cultures with other cell types.

3:

Depending on the age of the cartilage donor, the incubation time with collagenase may have to be adjusted. Younger cartilage (neonatal to infant) may only need 4--6 hours to be completely digested, in which case the reaction needs to be stopped. In our experience, neonatal cartilage is completely digested after 4 hours. In contrast, adult cartilage needs an overnight digestion.

4:

Depending on the subtype of human cartilage (auricular, costal, nasoseptal and articular), different enzymatic digestion sequences may be used:

a. An alternate digestion protocol (from Bradham *et al.*) for articular cartilage (All in DMEM-LG at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% air):
    1. Digest in 2 mg/ml hyaluronidase (45 min),
    2. Digest in 2 mg/ml trypsin (45 min)
    3. Digest in 4 mg/ml collagenase II for 2--3 hours

b. A further alternate digestion protocol (from Islam *et al.*) (All in F12 medium at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% air):
    1. Digest in 0.15% (w /v) pronase for 1 h
    2. Digest in 0.15% (w/v) Clostridium histolyticum collagenase overnight
c. Another alternate digestion protocol
    1. While collecting the cartilage, digest in Hyaluronidase 0.05% in PBS
    2. Transfer minced cartilage to 0.2% trypsin for 45 minutes at 37 °C for 45 minutes with stirring.
    3. Discard the trypsin solution
    4. Digest the cartilage slices in 10 mL of 0.2% collagenase solution
    5. Retain this suspension in a sterile 50-mL polypropylene tube
    6. Add fresh 0.2% collagenase solution to the cartilage and digest for another 45 min at 37°C with stirring.
    7. Retain this suspension as well, and pool with the first suspension.
d. For nasoseptal cartilage (from Aigner *et al.*): Perform enzymatic digestion in: 2 mg/ml collagenase type II, 0.1 mg/ml testicular hyaluronidase and 0.15 mg/ml DNAse (Fluka, USA) in DMEM-LG at 37 ºC in a humidified atmosphere of 5% CO2 and 95% air for 18 hours.
e. For auricular cartilage: Perform enzymatic sequential digestion with:
    a. 0.1% trypsin (30 minutes),
    b. 0.1% hyaluronidase (60 minutes)
    c. 0.1% collagenase type II (overnight)
    d. 0.15mg/ml DNAse (optional)

    all in DMEM-LG at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% air

5:

To maximize cell yield, the remaining undigested material after the second digestion (step 10 from isolation protocol) can be plated on tissue culture plastic (plates/flasks) to allow outgrowth of any remaining cells.

6:

Serum lots vary in their ability to support chondrocyte grown. It is therefore important to screen several lots and purchase a batch of the best-performing serum.

7:

Although chondrocytes can be expanded well in serum-supplemented DMEM, after the first passage a change into a fibroblastic-like phenotype can be observed. To prevent this, a chondrocyte specific medium should be used. In our experience, chondrocyte medium (see materials above) provides a good proliferative support and chondrocyte phenotype maintenance based on the observation that articular cartilage markers expression is maintained compared to chondrocytes expanded in serum-supplemented DMEM.

8:

Chondrocytes can produce and deposit large quantities of ECM, especially when they are at high density. In this case, trypsin will not work very efficiently. To improve detachment, Accutase® solution (Sigma-Aldrich) can be used. This solution contains proteolytic and collagenolytic enzymes that work well in detaching cells with high production of ECM. Accutase® may also be useful if trypsin-sensitive cell surface epitopes must be preserved (*e.g.*, for immunophenotyping).

9:

Polypropylene 96 well plates can be difficult to find pre-sterilized. They do survive autoclaving.

10:

For aggregate culture in 96-well plates, it is useful to note that the medium in the peripheral wells evaporates faster than in the rest of the plates. Depending on the experiment, it may be useful to not seed these wells, but rather fill them with plain medium or di-water

11:

Alternate base media for chondrocyte culture include Ham's F12 or DMEM/F12 1:1