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Chapter 11

Encapsulation of Human Articular Chondrocytes into 3D Hydrogel: Phenotype and Genotype Characterization

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Abstract

This chapter is intended to provide a summary of the current materials used in cell encapsulation technology as well as methods for evaluating the performance of cells encapsulated in a polymeric matrix. In particular, it describes the experimental procedure to prepare a hydrogel matrix based on natural polymers for encapsulating and culturing human articular chondrocytes with the interest in cartilage regeneration. Protocols to evaluate the viability, proliferation, differentiation, and matrix production of embedded cells are also described and include standard protocols such as the MTT and [3H] Thymidine assays, reverse transcription polymerase chain reaction (RT-PCR) technique, histology, and immunohistochemistry analysis. The assessment of cell distribution within the 3D hydrogel construct is also described using APoTome analysis.

Key words: Natural polymers, Hydrogels, Gelation, Chondrocyte encapsulation, Cell culture and survival within 3D matrix, Cell-based technology, Cartilage regeneration

1. Introduction

Cell encapsulation technology holds promise in many areas of medicine and biotechnology. For example, some important applications are treatment of diabetes, production of biologically important chemicals, and controlled release of drugs. The hope that encapsulated cells might be used as a therapeutic strategy is increasingly being realized and is expected to have enormous potential in medicine in the near future. However, to be a viable complement to the current methods of cell transplantation therapy, encapsulation technology has to fulfill the strict requirements applicable to these types of therapeutic strategies, such as

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performance, biosafety, biocompatibility, stability, availability, purity, characterization, and cost (1). In a typical encapsulation process, cells are suspended in a liquid solution, which is then rapidly solidified or gelled to entrap cells in the matrix. It is widely understood that in order to encapsulate living cells within an artificial matrix, the encapsulating conditions must not damage the living cells and the matrix material must be biocompatible and at the same time provide a natural microenvironment for the cells to sustain their viability, function, and growth or differentiation. Additionally the mechanical strength of the encapsulating matrix is critical, particularly when long-term implantation is envisaged. The mechanical strength is essential for maintaining the matrix integrity and to withstand manipulations associated with in vitro culture, implantation, and in vivo existence. Moreover, it is essential that the matrix should have suitable diffusion properties to ensure sufficient access of nutrients, for encapsulated cells to remain viable and functional, and the removal of secreted metabolic waste products. Thus, there is a need for relatively mild cell encapsulation methods, which offers control over properties of the encapsulating matrix.

An exciting approach for cell delivery is the use of materials that can undergo a gelation process that is cell-compatible and can be injected into the body (i.e., hydrogels). This approach enables the clinician to transplant the cell and polymer combination in a minimally invasive way (2). Hydrogels are appealing for cell delivery because they are highly hydrated three-dimensional networks of polymers that provide a place for cells to adhere, proliferate, and differentiate and they can often be processed under relatively mild conditions. Gelation can occur through a change in temperature (thermoreversible gels), or via chemical crosslinking, ionic crosslinking, or formation of an insoluble complex. Covalent crosslinking is a common method to precisely control the crosslinking density of hydrogels. However, the toxicity of cross-linking molecules must be considered. Many chemical crosslinking agents negatively impact cell viability and cause reactions in vivo, precluding the use of most chemically crosslinked gels for encapsulation. Ionic crosslinking with multivalent counterions is a simple way to form hydrogels. However, those ions could be exchanged with other ionic molecules in aqueous environments, resulting in an uncontrolled deterioration of the original properties of hydrogels.

Multicomponent blends of synthetic, semi-synthetic, and naturally occurring macromolecules have been explored for the encapsulation of a variety of mammalian cell types (3–5). Naturally-derived polymers are abundant, biodegradable, and usually undergo gelation under gentle conditions. In addition, they have shown low toxicity. Natural polyanions include several polysaccharides (e.g., alginate, carboxymethyl cellulose,

carrageenan, cellulose sulfate, gellan gum, gum arabic, heparin, hyaluronic acid, xanthan, dextran sulfate) and chitosan, the only naturally occurring polycation.

Although alginate-based materials are still receiving a great deal of attention, a wide variety of polyelectrolyte gels and complexes have now been investigated for cell encapsulation. Alginate, a negatively-charged polysaccharide, can either be used alone, or in conjunction with positively-charged polylysine to form alginate–polylysine polyelectrolyte complexes. Alginate itself may be ionically crosslinked by divalent cations such as calcium and barium. Although the encapsulating method based on ionic crosslinking of alginate (a polyanion) with polylysine or polyomithine (polycation) offers relatively mild encapsulating conditions and quite stable matrices, their mechanical properties and long-term stability are poor. Moreover, these polymers when implanted in vivo, are susceptible to cellular overgrowth, which restricts the permeability of the matrices to nutrients, metabolites, and transport proteins from the surroundings, leading to starvation and death of encapsulated cells.

Other encapsulation matrices have been tested and they were either not sufficiently mechanically stable or suffered from other surface or matrix related deficiencies such as shrinkage in either PBS or in culture media. In order to overcome these limitations, several approaches have been considered and tested, like crosslinking, chemical adjustment of charge density, combination of low and high molecular weight polyelectrolytes, adjustment of osmotic pressure, polymer grafting, polymer blending, among others.

Currently, hydrogels are being used in an attempt to engineer a wide range of tissues, including cartilage, bone, muscle, fat, liver, and neurons. Hydrogels have a similar macromolecular structure to cartilage, which is a highly hydrated tissue composed of chondrocytes embedded in type II collagen and glycosaminoglycans. Thus, cartilage is an obvious tissue to engineer using hydrogel matrices. Numerous hydrogel systems embedded with chondrocytes have been developed and tested both in vitro and in vivo.

Carrageenans are high molecular weight sulfated polygalactans derived from several species of red seaweeds (*Rhodophyceae*). The most common forms of carrageenan are λ, κ and ι. Carrageenan has alternating disaccharide units composed of d-galactose-2-sulfate and d-galactose-2,6-disulfate, being the galactose residues joined by -1,3 and -1,4 linkages. Carrageenans resemble to some extent the naturally occurring glycosaminoglycans owing to their backbone composition of sulfated disaccharides. The different composition and conformation of carrageenans results in a wide range of rheological and functional properties. Carrageenans form thermoreversible gels. The polysaccharide is water soluble when heated and gels upon cooling the solution in presence of electrolytes (Ca^{2+} and K^{+}). κ-carrageenan gels in the presence

of K^+ ions to form strong crisp gels, whereas ι-carrageenan gels in the presence of Ca^{2+} ions to form elastic gels (6). Carrageenans have been used extensively in the food industry. In addition to incorporation in foods, carrageenan has been used as an ingredient in pharmaceuticals and personal care products, such as toothpaste and cosmetics (7).

The use of carrageenan has not been fully explored as potential biomaterial and we believe that the structural diversity of carrageenans can provide very interesting rheological and functional properties in cell encapsulation technology. Because of its widespread use in food and pharmaceutical industry, purified carrageenan materials are readily available with reliable, predictable, and chemically defined composition.

The aim of the present chapter is to provide experimental procedures to prepare a hydrogel-based matrix for the encapsulation of chondrocytes, and evaluate its potential as a cell-delivery carrier for cartilage regeneration strategies. We focus on methods for culturing and expanding human chondrocytes obtained from articular cartilage biopsies and evaluating the maintenance of cell viability and function within the 3D hydrogel matrix. Characterization methods to evaluate other properties of the hydrogel system, such as hydrogel strength and stability, gelation kinetics, and hydrogel permeability are also of central importance, but these topics will not be described in this chapter.

2. Materials

2.1. Hydrogel

1. Carrageenan polysaccharides (ι- and κ-carrageenans both from Fluka Biochemika, Denmark).
2. Human fibrinogen and human thrombin (Baxter AG, Vienna, Austria) plus the syringe holder – Duploject. The syringe holder allows (with a single press) for an injectable hydrogel to be formed with an equal distribution of both components.
3. High molecular weight hyaluronic acid from *Bacillus subtilis* (kindly provided by Prof. Hilborn, Uppsala University).
4. Double distilled water, $CaCl_2$ and KCl powders to trigger gel formation.

2.2. Cell Culture: Primary Culture of Human Articular Cartilage Biopsies

1. Coon's F-12 Modified Ham's medium (Biochrom A.G. Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Invitrogen Life Technologies, Carlsbad, CA).
2. Trypsin solution (0.25%) with ethylenediamine tetraacetic acid (EDTA) (1 mM) (Invitrogen Life Technologies, Carlsbad, CA).

3. Collagenase I, collagenase II (both from Worthing Biochemical, Lakewood, NJ), hyaluronidase (Sigma, St. Louis, MO), and trypsin (Invitrogen Life Technologies, Carlsbad, CA). Store at −20°C.

2.3. Cell Culture: 2D Monolayer Cell Expansion

1. Coon's F-12 Modified Ham's medium supplemented with 10% fetal calf serum.
2. Serum free medium (8) freshly prepared twice a month in accordance with the composition listed in Table 1. Human recombinant growth factors (Austral Biologicals, San Ramon, CA, USA) and insulin (Sigma-Aldrich, Steinheim, Germany).
3. Trypsin solution (0.25%) with ethylenediamine tetraacetic acid (EDTA) (1 mM).

2.4. Cell Culture: 3D Cell Encapsulation and Culture Under Chondrogenic Differentiation

1. Agarose working solution 1% (w/v) in PBS (1×). Transfer this solution to a glass bottle and autoclave to ensure adequate sterilization. Store at room temperature.
2. Plastic ring containers (made, for example, of 2 mL plastic pipettes cut in equal parts, or pre-made plastic rings used in

Table 1
Serum free medium composition (adapted from Malpeli et al. (8))

Component (protein/growth factor)	Final concentration
Apo-transferrin	25 μg/mL
Ascorbic acid	250 μM
Biotin	33 μM
Cholesterol	13 μM
Dexamethasone	10 nM
Epidermal growth factor	5 ng/mL
Fibroblast growth factor 2	5 ng/mL
Holo-transferrin	25 μg/mL
Human serum albumin	1% (w/v)
Insulin	10 μg/mL
Linoleic acid	4.5 μM
N-acetylcysteine	50 μM
Platelet derived growth factor	5 ng/mL
Sodium pantothenate	17 μM
Sodium selenite	30 nM

cell biology laboratories to collect cell clones) to be used as a mold for the injectable hydrogel (to give form and shape). Choose an appropriate size to determine the volume of the hydrogel and sterilize by autoclaving.

3. Chondrogenic medium (9): Coon's F-12 Modified Ham's medium supplemented with 6.25 μg/mL Bovine Insulin, 6.25 μg/mL Human Apotransferrin, 1.25 μg/mL Linoleic Acid, 5.35 μg/mL Bovine Serum Albumin, 1 mM sodium pyruvate, 50 μg/mL Ascorbic acid, 10 ng/mL Transforming Growth Factor β-1 (TGF β-1), and 10^{-7} M dexamethasone.

2.5. Viability and Proliferation of Encapsulated Cells

1. Thiazolyl Blue staining (MTT; Sigma-Aldrich) powder dissolved at concentration of 5 mg/mL in freshly prepared phosphate buffer solution (PBS) (1×). Store aliquots at −20°C.
2. Spectrophotometer (Beckman Du® 640, USA).
3. 1450 Microbeta – Liquid Scintillation & Luminescence Counter (Wallac, Triliux, USA).
4. [Methyl-^{3}H] Thymidine (Amerstam, GE Healthcare, UK) solution. Store at 2°C (see Note 1).
5. EcoLite™ – Scintillation Liquid (JCN, Costa, CA, USA). Store at room temperature and protect from light.
6. DNA buffer solution (Invitrogen, Carlsbad, CA, USA). Store at 4°C.
7. Washing solutions, absolute ethanol, and PBS (1×). Store these solutions at room temperature.

2.6. ApoTome Image Acquisition

1. Axiovert 200M microscope equipped with the ApoTome module and the AxiocamHR camera (Carl Zeiss, Jena, Germany) as well as Axiovision Software (Carl Zeiss, Jena, Germany).
2. Hoechst 33342 at final concentration of 5 μg/mL in PBS (1×). Store at 4°C and protect from light.

2.7. Histology and Immunohistochemistry Analysis

1. Graded ethanol solutions (70, 90, 95, and 100%); xylene and wax.
2. 4% Formaldehyde solution in PBS. Store aliquots at −20°C.
3. Methanol/H_2O_2 solution: methanol/H_2O_2 (49:1, v/v). Store at 4°C.
4. Sodium acetate solution: 50 mM Na Acetate, pH 5.0. Adjust the pH solution with HCl (0.05 M) or with NaOH (0.05 M).
5. Peroxidase substratum solution: 3-amino-9-ethylcarbazole 0.04% in dimethylformamide, 45 mM Na Acetate (pH 5), 0.03% H_2O_2 (100:900:1). Store at room temperature and protect from light.

6. Harris's haematoxylin solution (Bio Optica, Milano, IT).
7. Mounting medium: Gel mount (Biomeda Corp., Foster City, CA, USA).

2.8. mRNA Extraction

1. PCR supplies (microcentrifuge tubes, PCR tubes, PCR plates, pipette tips) and water (diethylpyrocarbonate (DEPC) treated water) free of DNAase/RNAase.
2. Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Store at 4°C.
3. Homogenizer (Ultra Turrax® Model T25, IKA Labortechnik GMbH, Germany).
4. 2-Isopropanol.
5. Solution of chloroform/isoamyl alcohol (49:1) (v/v). Store at room temperature.
6. 70% Ethanol solution. Store at −20°C.

3. Methods

3.1. Hydrogel Preparation

1. Weigh out 0.6 and 0.4 g of ι- and κ-carrageenans, respectively, and place them inside appropriate tubes to be autoclaved (120°C for 20 min) to ensure sterility. Sterilize double distilled water, glassware, and lab tools (e.g., two or three beakers, magnetic stirrers, and forceps) by autoclaving them prior to use.
2. Under sterile conditions (laminar flow cabinet), heat 50 mL of double distilled water (covered with a cap to avoid evaporation) up to 65°C and dissolve the carrageenan powder under constant stirring until forming a homogenous preparation.
3. When the solution is clear, homogenous, and without bubbles, switch off the stirrer and the heater power. Leave it at room temperature. Transfer the polymeric solution to a 50-mL tube. The solution is ready to be used. It can remain at room temperature from days to weeks until further use.

3.2. Human Articular Chondrocytes Primary Culture

1. Clean human articular cartilage biopsy samples from connective and muscular tissues and/or subchondral bone and cut them in small fragments (see Note 2).
2. Wash articular cartilage fragments several times with freshly prepared PBS (1×) and centrifuge in PBS (1×) at 180 × *g* for 5 min.
3. Discard the supernatant and refill with the enzymatic cocktail solution made of: trypsin 0.25% (v/v), collagenase I (400 U/mL), collagenase II (1,000 U/mL), and hyaluronidase (1 mg/mL). Place in a thermostatic bath at 37°C for 30 min.

4. Collect the supernatant and block the enzymatic activity with Coon's modified ham's medium with 10% fetal calf serum (FCS). Centrifuge for 10 min at $180 \times g$.
5. Plate the cell suspension under anchorage-dependent conditions: plastic Petri dishes coated with Coon's modified ham's medium with 10% of FCS.
6. Repeat steps 3–5 until no biopsy material is available. Have in mind that this procedure can take from 2/3 h up to 1 entire day, depending on the amount of biopsy material. Therefore, prepare, at least 1 day before, chemicals and materials (aliquots of the different enzymatic solutions, solutions, several bistouries, and forceps) necessary to perform this procedure.
7. Keep the cells in culture under conditions described in step 5 for 3/4 days. Trypsinize cells and perform an initial count (counting is necessary to report cell duplication number relative to the starting number of primary cells). The next step is to re-plate the cells under serum-free medium conditions.

3.3. 2D Monolayer Cell Expansion

1. Before starting the 2D monolayer expansion of human articular chondrocytes under serum-free medium conditions, it is important to be aware that all growth factors should be previously aliquoted and ready for use. The medium used during all the expansion period of a primary culture should be always from the same batch of aliquots. This is important for reducing variability between independent experiments.
2. Enzymatically detach and count the cells (reported as step 7 in Subheading 3.2). Use Coon's modified Ham's medium with 10% of FCS to block the activity of trypsin.
3. Wash three times with PBS (1×) the Petri dish coated with Coon's modified Ham's medium (plus 10% FCS) to remove any residual traces of serum. Re-plate the cells at a density of $4 \times 10^4/\text{cm}^2$ using serum free medium prepared according to the Table 1. Change the medium three times a week.
4. When cells have a sub-confluent distribution, repeat step 2 using Coon's modified Ham's medium with 1% of FCS instead of 10% FCS. Repeat step 3 until the cell number reaches the number necessary to start the experiment.

3.4. 3D Cell Encapsulation and Culture Under Chondrogenic Differentiation Conditions

1. Prepare aliquots of fibrinogen at a final concentration of 3,000 UIK/mL, as well as thrombin (500 IU/mL). Add hyaluronic acid powder to the latter solution to obtain a concentration of 0.75% w/v, and allow the polymer to dissolve overnight at 4°C under gentle shaking.
2. At the end of in vitro cell expansion, detach enzymatically the cells and count them. Calculate the number of hydrogels to be prepared (with and without cells) based on the final number

of cells available for encapsulation and the cell density within the 3D hydrogel.

3. Prepare a medium that will trigger the gel formation of carrageenan solution. For that, dissolve $CaCl_2$ (0.265 g/L) and KCl (0.4 g/L) in Coon's modified Ham's medium and render this solution sterile by syringe filtration (0.22-μm filter).

4. Add fibrinogen solution 35% (v/v) to the carrageenan polymeric solution. Mix them well with a pipette avoiding the formation air bubbles (solution B). Make the cell suspension in the medium prepared in step 3 and add thrombin solution to a final concentration of 5.9% (v/v) (Solution A) (see Note 3). The cell number depends on the final cell density within the hydrogel (i.e., 2×10^6 cells/mL). See Fig. 1.

5. Load solution A and B in to two separate 1-mL syringes (see Fig. 1). Place them with care in the Duploject double syringe holder and set the connector at the syringe extremity end. Insert the needle and be ready to fill the plastic ring molds for giving shape and size to the injectable hydrogel.

6. Mix well before starting the injection to avoid sinkage of cells to the bottom of the syringe by gravity. Inject the same volume

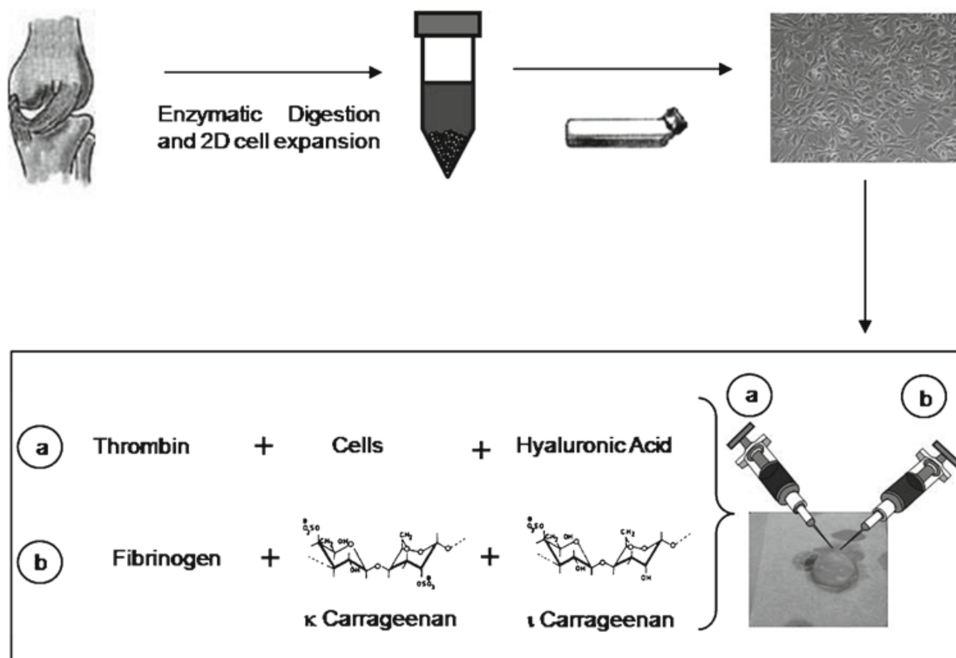

Fig. 1. Schematic representation of the method to form the hydrogel. From the *top left* (following the *arrows*): human articular chondrocytes biopsy and successive digestions; Light microscopy image of primary monolayer culture in serum free medium during the 2D expansion period; biodegradable hydrogel system with the content composition in both syringes (a and b) and the macroscopic morphology of the formed gel. Reproduced with permission from Pereira et al. (14).

of hydrogel into the rings within a 24 multi-well plate (previously coated with agar).

7. Wait 5–10 min and turn the gels upside down to allow a better cell distribution within the hydrogel (to avoid cells sinking to the bottom of the gel by gravity). Wait another 5–10 min. Meanwhile prepare the chondrogenic medium. Calculate the volume necessary to be prepared knowing that the total volume of the hydrogel must be covered with medium.
8. Discard the plastic rings and add medium to each well. Place the 24 multi-well plate in to an incubator (37°C and 5% CO_2). Change the chondrogenic medium three times a week.

3.5. In Vitro Viability and Proliferation of Encapsulated Cells

It is necessary for each time point to evaluate the viability and proliferation rates of the human articular chondrocytes encapsulated within the gel. Repeat the procedure described below: MTT assay corresponding to the viability analysis (10) and [methyl-^{3}H] Thymidine (11) to proliferation.

3.5.1. MTT Assay (Viability)

1. Prepare serum-free medium with MTT at a final concentration of 50 μL/mL. The final volume depends not only on the number of samples to be analyzed (remember to perform the analysis at least in triplicate) but also on the volume occupied by the construct. All individual samples must be completely immersed. The volume used for all the samples must be the same, even if their volume is different.
2. At pre-determined time points, carefully remove the medium from the well in which the 3D construct was kept during the in vitro culture without damaging it.
3. Refill the well with recently prepared medium described in step 1. Incubate (37°C and 5% CO_2) for 2–3 h protected from light.
4. Collect the medium and add 1 mL absolute ethanol. The hydrogel with encapsulated cells will form blue dots (the dots correspond to the cells). Break up the hydrogels by pipetting the construct up and down. The same number of movements should be applied to all the conditions.
5. For each sample, collect 1 mL of liquid into separated tubes and measure the dye absorbance at 570 nm with background subtraction at 670 nm in a spectrophotometer.
6. Repeat this procedure for remaining time points programmed for the experiment and at the end plot the absorbance values versus time.

3.5.2. [Methyl-^{3}H] Thymidine Assay (Proliferation)

1. Prepare serum free medium with [methyl-^{3}H] Thymidine at a final concentration 1 μCi/mL. Before handling radioactive material, ensure you are familiar with good radiation safety

practice (see Note 1). Place all materials in contact with the radioactive material in a closed bag and place in a designated radioactive waste container for disposal.

2. Remove the medium from the sample for analysis. Incubate the 3D constructs (37°C and 5% CO_2) with the medium prepared in step 1 for 16 h ensuring that the hydrogels are completely immersed in the medium.
3. After the incubation period, collect the medium in a closed flask and place it in a container (designated for radioactive solutions). Wash the samples several times with PBS (1×) to remove residual [methyl-^{3}H] Thymidine (i.e., not incorporated into cellular DNA) from the 3D hydrogels. Add these solutions to the flask containing [methyl-^{3}H] Thymidine.
4. Add 500 μL of DNA buffer solution to the 3D hydrogel and break up the hydrogel by pipetting up and down, according to the protocol used for the MTT assay (MTT, step 4). Recover the supernatant and store at −20°C.
5. After collecting the samples for all the experimental time points, thaw the frozen samples and transfer 400 μL of the solubilized-cell solution into 4 mL scintillation vials and count. Specify a volume for each and add 2 mL of scintillation liquid. Transfer the solution to the Beta Counter containers and read. The samples must be performed in triplicate for each condition as a minimum. Plot the values versus time. Cell proliferation is evaluated by [methyl-^{3}H] Thymidine incorporation, which correlates to cell number as a function of culture time.

3.6. APoTome Analysis

This is an epifluorescent illumination technique (12, 13), which allows images to be obtained throughout most of the hydrogel without the need for any manipulation procedures, such as dehydration for histology (which may introduce artifacts). This can be a powerful tool for in vitro cellular characterization (distribution and viability) of hydrogels.

1. Carefully aspirate the medium from the well where the samples to be analyzed are located.
2. Incubate (37°C and 5% CO_2) the samples with a serum-free medium containing Hoechst 33342 at a final concentration of 5 μg/mL for 30 min (and protect from light).
3. Transfer the hydrogel to a small Petri dish (3 mL) and start to acquire images by epifluorescent illumination using an Axiovert 200M microscope equipped with the ApoTome module. Set up the camera and select the correct filter for the required channel. Start the image acquisition stacking optical sections and subsequent 3D reconstruction using the Axiovision Software (see Fig. 2). During this procedure,

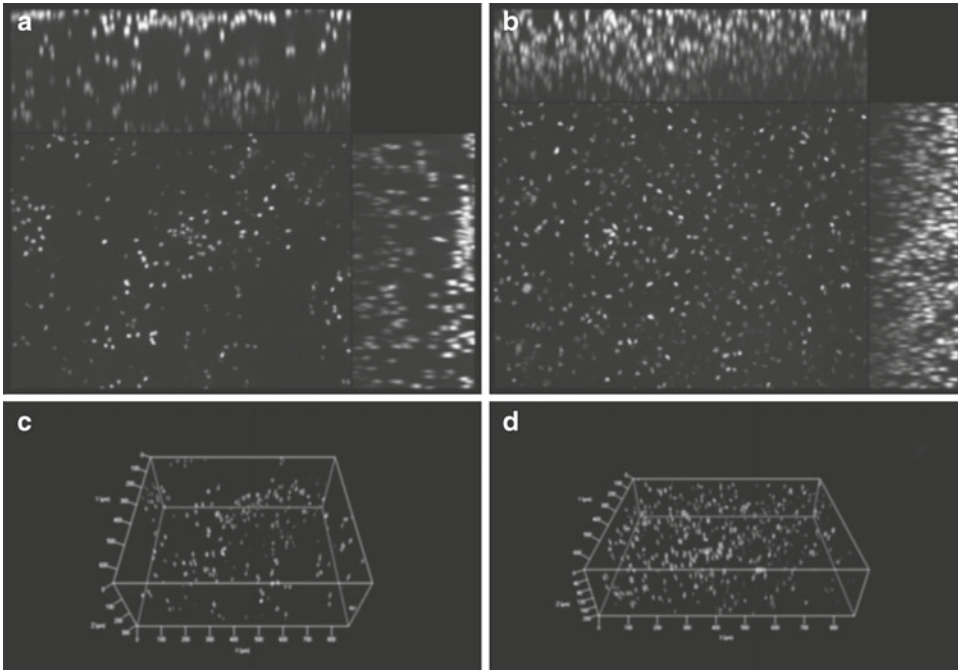

Fig. 2. Representative images of live human articular chondrocytes encapsulated within the hydrogel at 1 (**a**, **c**) and 3 weeks (**b**, **d**) of culture after labeling of the cell nuclei with Hoechst and optical sectioning by structured epifluorescent illumination. An image projection along the *X*, *Y*, and *Z* axes (**a**, **b**), and their respective 3D reconstructed stacks (**c**, **d**). Reproduced with permission from Pereira et al. (14).

try not to overexpose the sample to the fluorescent light (to prevent photo bleaching). Afterwards, samples can be processed for histology.

3.7. Histology and Immunohistochemistry Analysis

1. Discard the medium, rinse the sample three times with PBS (1×) and transfer the sample to a test tube. Add 10 mL of 4% formaldehyde solution and incubate for 6 h under rotation conditions. Make sure that the tube is tightly closed to avoid possible leaks.
2. Dehydrate the samples by immersing them in a graded series of ethanol solutions (70, 90, 95, and 100%) for 30 min each and xylene (10 min). Due to the high water content of the hydrogel, sample shrinkage is normally observed during dehydration (due to removal of the intrinsic water in the sample). Be careful not to lose any sample during the dehydration steps.
3. Embed the hydrogels in paraffin. Perform a series of different melting points on the paraffin specifically at 45, 55, and 60°C (each one for 45 min). Perform the final paraffin inclusion and keep the sample at −20°C.

4. With the help of a microtome, cut sample sections with a thickness of between 4–6 μm. Dewax the sections repeating step 2 in reverse in order to perform any specific staining steps (e.g., Harris's haematoxylin and Eosin, Toluidine Blue) or immunohistochemistry.

3.7.1. Immunohistochemistry

1. After step 4, incubate the cut sections with methanol/H_2O_2 (49/1) solution for 30 min to inhibit endogenous peroxidases.
2. Discard the solution and incubate with hyaluronidase solution for 20–30 min. Wash several times with PBS (be careful to not detach the sample from the glass).
3. To reduce non-specific binding, incubate with goat serum for 1 h. Wash three times with PBS. Incubate with a specific primary antibody for 1 h at room temperature.
4. Wash the sections three times with PBS and incubate with secondary biotinylated anti-mouse IgG and peroxidase-conjugated egg-white avidin. Wash the sections with PBS and add peroxidase substrate solution. Incubate at room temperature (protected from daylight) for a period of 15 min.
5. Counterstain the sample with Harris's haematoxylin. Mount the section with gel mount and observe by optical microscopy.

3.8. mRNA Extraction/Isolation

1. Collect the medium from the wells and wash the 3D hydrogels several times with PBS.
2. Aspirate the hydrogel carefully with the help of a pipette and place in a tube.
3. Add 1 mL of Trizol and place immediately on ice.
4. Homogenize the samples at 15,000 × *g* for 30 s, rinsing thoroughly the probe with DEPC water.
5. Transfer 1 mL of sample to a 1.5-mL tube. Incubate at room temperature for 5 min. Add 200 μL of chloroform/isoamyl alcohol (49:1) and incubate the samples at room temperature for 4 min.
6. Centrifuge at 12,000 × *g* for 10 min at 4°C.
7. Collect the supernatant into a new 1.5-mL microcentrifuge tube. Be very careful not to disturb the lower part of the gradient solution, to minimize possible protein contamination.
8. Add an equal volume of 2-isopropanol per tube (1:1). Hand mix and incubate at room temperature for 10 min.
9. Centrifuge at 12,000 × *g* for 10 min at 4°C. At this stage, a pellet will form in the bottom of the tube. Be aware of possible difficulties for visualizing the pellet due to its small size and irregular shape.

10. Discard the liquid and add 1 mL of 70% ethanol. Centrifuge at 7,500 × *g* for 5 min at room temperature.
11. Carefully remove the ethanol solution and dissolve the RNA pellet in water (50–100 μL RNAase-free water). The RNA of each sample is ready to be quantified and consequently used in a RT-PCR.

4. Notes

1. [Methyl-^{3}H) Thymidine is a radioactive reagent with an approximate 15 day decay period. When working with radioactive chemicals, it is important to follow correct local and national safety guidelines. This will include the use of personal protective equipment (e.g., always work with gloves and use two pairs if possible) and the disposal of radioactive waste (radioactive liquids and other materials in contact with radioactive chemicals), which must be via designated containers for radioactive waste disposal.
2. The importance of protocol design in yielding cells of adequate quantity and quality is paramount for the success of this technology. Therefore, several variables should be considered when performing any type of primary culture (i.e., the total final number of cells enzymatically obtained during the tissue digestion and its biological quality). During the first step, it is very important to clean the biopsy sample from all adherent tissues (muscular, connective, and bone) to avoid possible contamination of cartilage cells. The biological quality of the primary culture will depend on the biological functionality of the tissue (cartilage in this case) and also on the degree of tissue purity.
3. Having a homogeneous distribution of cells in the syringe before the injection of the hydrogel is extremely important. Be aware that by gravity cells in suspension will sink to the bottom of the gel. To avoid that, shake the syringe and try to keep it horizontal before injection. In this way, the cell distribution within the hydrogel construct will be more uniform. Even knowing that this is an easy to handle injectable hydrogel, with in situ gelling properties, try to minimize any spill to avoid wasting cells and polymer solution.

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