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CELLULAR MICROCOMPARTMENT AND PREPARATION METHODS

Description

The invention relates to a cellular microcompartment for preserving the pluripotent state of human cells. The invention also relates to a preparation
5 method for obtaining such three-dimensional (3D) cell culture compartments.

Pluripotent cells are considered an important human cellular resource, and their cultivation is of growing interest, particularly in the medical and pharmaceutical fields. Thus, obtaining pluripotent cells in large quantities would make it possible to meet the new needs expressed by the pharmaceutical industry, which is
10 tending to reduce the use of animal models and to use cell models that are more relevant than the numerous cell lines currently in use. High-throughput tests developed by pharmaceutical companies already use large quantities of human pluripotent cells. Similarly, tissue engineering and cell therapy in humans depend on the availability of industrial quantities of human pluripotent cells.

15 At present, human pluripotent cells are mainly grown in a two-dimensional (2D) environment, such as petri dishes, far removed from the 3D environment in which the cells normally live. Handling these 2D-cultured cells is often tricky, requiring purification steps, enzymatic release, and so on. In addition, these cells are difficult to preserve and have a very low survival rate when frozen. The
20 possibility of sending frozen pluripotent cell cultures in massive quantities, compatible with liquid culture, by conventional freight represents a major challenge for both research laboratories and the pharmaceutical industry.

In response to this situation, three-dimensional culture systems have been developed to increase the throughput, efficiency, and quality of human
25 pluripotent stem cell culture systems.

However, existing 3D culture systems are not entirely satisfactory. Uncontrolled fusion phenomena are often observed, giving rise to cell aggregates whose size ($>200\text{ }\mu\text{m}$ in diameter) prevents adequate diffusion within the culture medium. In such 3D culture systems, cell differentiation is difficult to control and/or the rate of cell death is very
30 high. Generally speaking, the lack of homogeneity of the products resulting from 3D

cell culture, as well as the cost of such techniques, make this technology uncompetitive compared to 2D culture, which is nevertheless unsatisfactory.

For example, Ashida et al. "Propagation of Human iPS cells in alginate-based microcapsules prepared using reactions catalyzed by horseradish peroxidase and catalase" Artificial cells, Nanomedicine and Biotechnology 2015 Early Online 1-4, describes aggregates of cells in a hydrogel. However, the cell proliferation is very low and pluripotency is not assured in the solution proposed in that article.

There is therefore a need for a 3D cell culture system that can provide large quantities of pluripotent cells with controlled phenotypes, which can be easily used for both basic research and industrial applications.

Summary of the invention

By working on the development of cellular microcompartments for cell culture in 3D, the inventors developed a system that enables the mass culture of human pluripotent cells in a liquid suspension, while maintaining their phenotype. The microcompartments that develop allow cells to be grown in a liquid medium, using the media traditionally used in 2D culture, while protecting the cells and controlling their phenotype to avoid spontaneous differentiation and maintain pluripotency. More precisely, the microcompartments, or capsules, developed by the inventors comprise in succession, organized in a substantially homocentric manner, a hydrogel shell, a layer of extracellular matrix, and one or more layers of human pluripotent cells surrounding a central lumen. Unlike existing culture systems, the hydrogel shell of the capsules according to the invention protects the cells from mechanical stresses caused by collisions or fusions during culture in a liquid suspension. Particularly advantageous is the "cyst" structure of the microcompartments according to the invention, enabling them to be frozen with a high cell survival rate. The cells can also be differentiated before use, directly within the microcompartment, or used at the pluripotent stage, in both 3D and 2D cultures. The inventors have also developed methods for preparing such cellular microcompartments, ensuring that the cyst shape is obtained and maintained, which is conducive both to freezing and to controlling the phenotype of the cells they contain.

The object of the invention is thus a cellular microcompartment comprising successively, organized around a lumen:

- at least one layer of human pluripotent cells;
- an extracellular matrix layer;
- 5 - an outer layer of hydrogel,

said microcompartment being closed.

Advantageously, culture medium fills the spaces left between the layers.

Another object of the invention is a method for preparing a cellular microcompartment according to the invention, comprising the steps of

- 10 (a) incubating human pluripotent stem cells in a culture medium containing a RHO/ROCK pathway inhibitor;
 - (b) mixing the pluripotent stem cells from step (a) with an extracellular matrix;
 - (c) encapsulating the mixture from step (b) in a hydrogel layer;
 - (d) culturing the capsules obtained in step (c) in a culture medium containing
 - 15 a RHO/ROCK pathway inhibitor;
 - (e) rinsing the capsules from step (d) to remove the RHO/ROCK pathway inhibitor;
 - (f) culturing the capsules from step (e) for 3 to 20 days, preferentially for 5 to 10 days, in a culture medium without any RHO/ROCK pathway inhibitor, and optionally recovering the obtained cellular microcompartments.
- 20 A further object of the invention is a method for preparing a cellular microcompartment according to the invention, comprising the steps of
- (a) mixing human differentiated cells with an extracellular matrix and cell reprogramming agents;
 - (b) encapsulating the mixture from step (a) in a hydrogel layer;

(c) culturing the capsules from step (b) for 10 to 40 days, and optionally recovering the cellular microcompartments obtained.

Brief description of the figures

Figure 1: Photo (A) and schematic representation (B) of a cellular
5 microcompartment forming a cyst according to the invention (1: hydrogel layer; 2: extracellular matrix layer; 3: pluripotent cell layers; 4: lumen).

Detailed description

The object of the invention is a 3D cellular microcompartment comprising human pluripotent cells, in which the pluripotent state of the cells is preserved.

10 Cellular microcompartment

The cellular microcompartment according to the invention forms a cyst, whose hollow center, or lumen, is preferably aqueous. In the context of the invention, a "cyst" is a closed, hollow structure containing layers that are substantially homocentric, meaning that they are organized successively around the same
15 point, with the outer layer enveloping the matrix layer, which envelops the cell layer, which surrounds the lumen. Generally speaking, the pluripotent cells constituting the cyst are polarized. The polarity of these cells inside the cyst can be demonstrated by the TJP-1 or ZO-1 proteins, both located on the internal/apical surface of the layer of pluripotent cells, which adjoins the lumen.

20 The lumen is generated, at the time of the formation of the cyst, by the cells which multiply and develop on the extracellular matrix layer. Advantageously, the lumen contains a liquid, more specifically culture medium.

In the context of the invention, "hydrogel layer" means a three-dimensional structure formed from a matrix of polymer chains, swollen using a liquid,
25 preferentially water. Advantageously, the hydrogel used is biocompatible, that is to say it is non-toxic to the cells. Furthermore, the hydrogel layer must allow the diffusion of oxygen and nutrients in order to supply the cells contained in the microcompartment and to enable them to survive. For example, the hydrogel outer layer contains alginate. Preferentially, the outer layer comprises only alginate. In

the context of the invention, "alginate" refers to linear polysaccharides formed from β -D-mannuronate (M) and α -L-guluronate (G), salts, and derivatives thereof.

Advantageously, the alginate is a sodium alginate, composed of more than 80% G and less than 20% M, with an average molecular weight of 100 to 400 KDa (for
5 example: PRONOVA™ SLG100) and a total concentration of between 0.5% and 5% by mass. According to the invention, the layer of hydrogel is devoid of cells. In one embodiment of the cellular microcompartment according to the invention, the outer layer comprises alginate.

The extracellular matrix layer may contain a few cells. As the cyst forms, the cells
10 hollow out their space in the matrix and multiply, filling the microcompartment. The boundary between the extracellular matrix layer and the pluripotent cell layer may therefore not be perfectly clear-cut. On the surface in contact with the cell layer, the extracellular matrix may therefore contain a few pluripotent cells. Conversely, the surface of the extracellular matrix layer in contact with the hydrogel layer is cell-free.

15 The extracellular matrix layer is necessary for pluripotent cells to survive in the microcompartment and for the cyst to be created.

Preferably, the extracellular matrix layer forms a gel on the inner side of the hydrogel layer, i.e. the side facing the lumen of the microcompartment. The extracellular matrix layer comprises a mixture of extracellular proteins and compounds necessary for cell
20 culture, and more particularly pluripotent cell culture. Preferentially, the extracellular matrix comprises structural proteins, such as laminins containing sub-units α 1, α 4, or α 5, sub-units β 1 or β 2 and sub-units γ 1 or γ 3, entactin, vitronectin, collagen, and growth factors, such as TGF-beta and/or EGF. In one embodiment, the extracellular matrix layer consists of, or contains, Matrigel® and/or Geltrex®.

25 According to the invention, the cellular microcompartment contains one or more layers of human pluripotent stem cells. A pluripotent stem cell, or pluripotent cell, means a cell that has the capacity to form all the tissues present in the entire organism of origin, but without being able to form an entire organism as such.

In one particular embodiment, the encapsulated cells are pluripotent stem cells, such
30 as induced pluripotent stem (IPS) cells, MUSE ("Multilineage-differentiating Stress

Enduring”) cells found in the skin and bone marrow of adult mammals, or embryonic stem (ES) cells obtained without destroying the embryo from which they are derived.

In the context of the invention, “induced pluripotent stem cells” (iPSCs or IPSs) refer to pluripotent stem cells obtained by genetic reprogramming of

5 differentiated somatic cells, and displaying a morphology and potential for self-renewal and pluripotency partly similar to those of embryonic stem cells. These cells are in particular positive for pluripotency markers, such as staining with alkaline phosphatase and expression of the proteins NANOG, SOX2, OCT4 and SSEA3/4. Methods for obtaining induced pluripotent stem cells are well known to
10 the person skilled in the art, and are described in articles by Yu et al. (Science, 2007, 318 (5858): 1917-1920), Takahashi et al. (Cell, 2007, 131(5): 861-872) and Nakagawa et al. (Nat Biotechnol, 2008, 26(1): 101-106).

In the case of embryonic stem cells obtained without destroying the embryo from which they are derived, pluripotent stem cells are defined as cells derived from the

15 inner cell mass of the blastocyst, and which have the capacity to lead to the formation of all the body's tissues. The pluripotency of the embryonic stem cells can be evaluated by the presence of markers such as the transcription factors OCT4 and NANOG and surface markers such as SSEA3/4, Tra-1-60 and Tra-1-81. The embryonic stem cells are obtained without destroying the embryo from
20 which they originate, for example using the technique described in Chung et al. (Cell Stem Cell, 2008, 2(2): 113-117).

In one embodiment, the human pluripotent stem cells used for the microcompartments according to the invention are induced to pluripotency from somatic cells.

25 Advantageously, the cell layer contains at least 95% by volume, preferably at least 96%, 97%, 98%, 99%, of cells and matrix produced by said cells. The cells are essentially pluripotent. “Essentially” means that at least 90% of the cells contained in the cell layer are pluripotent cells, preferably at least 95%, 96%, 97%, 98%, 99%, 100%, are pluripotent cells.

Advantageously, the lumen of the cyst contains culture medium. In particular, any culture medium that allows pluripotent cells to be grown in a suspension can be used, including any culture medium conventionally used in 2D culture.

The cellular microcompartment is closed. It is the hydrogel outer layer that gives
5 the cellular microcompartment its size and shape. The microcompartment may have any form compatible with cell encapsulation.

Advantageously, the dimensions of the cellular microcompartment are controlled. In one embodiment, the cellular microcompartment according to the invention has a spherical shape. Preferably, the diameter of such a microcompartment is
10 between 10 μm and 1 mm, more preferably between 50 μm and 500 μm , even more preferably less than 500 μm , preferentially less than 400 μm .

In another embodiment, the cellular microcompartment according to the invention has an elongate shape. In particular, the microcompartment can be ovoid or tubular in shape. Advantageously, the smallest diameter of such an ovoid or tubular
15 microcompartment is between 10 μm and 1 mm, more preferably between 50 μm and 500 μm , even more preferably less than 500 μm , preferentially less than 400 μm . "Smallest dimension" means twice the minimum distance between a point on the outer surface of the hydrogel layer and the center of the microcompartment.

In a particular embodiment, the thickness of the hydrogel outer layer is 5 to 40%
20 of the microcompartment radius. The thickness of the extracellular matrix layer represents 5 to 80% of the radius of the microcompartment and is advantageously bonded to the inner face of the hydrogel shell. The thickness of the pluripotent cell layer is around 10% of the microcompartment radius. The pluripotent cell layer is in contact with the extracellular matrix layer at at least one
25 point, and a space filled with culture medium may be present between the matrix layer and the cyst. The lumen then represents 5 to 30% of the microcompartment radius. In the context of the invention, the "thickness" of a layer is the dimension of said layer extending radially from the center of the microcompartment.

In one particular example, the cellular microcompartment has a spherical shape
30 with a radius of 100 μm . The hydrogel layer is between 5 μm and 40 μm thick. The extracellular matrix layer ranges in thickness from 5 μm to around 80 μm . The

pluripotent cell layer is 10 to 30 μm thick, with the lumen having a radius of around 5 to 30 μm .

Generally speaking, the presence of the hydrogel outer layer imposes a maximum size on the cell layer and, through confinement, limits uncontrolled cell proliferation, which could lead to cell death due to anoxia and/or uncontrolled cell differentiation in the deepest layers, i.e. those closest to the lumen of the cyst. In 2D, on a Petri dish, colonies are disks: Cells at the heart of the disk tend to die (each new cell resulting from a division is excluded from the colony by the lack of space) or to differentiate as a result of the stress from the surrounding cells; cells at the edge tend to differentiate, and only a band at the right distance away has the optimal phenotype. The topology of the microcompartment presented here, the inner surface of the sphere formed by the capsule, makes it possible to generate a "colony" of stem cells (the pluripotent cell layer) "without edges" where all the cells are optimally and equivalently positioned both for the diffusion of small molecules and in terms of mechanical stresses. Advantageously, the cell density in the microcompartment is between 1 and several thousand cells per microcompartment, preferably between 50 and 1000 cells per microcompartment of 100 μm radius.

Methods for preparing cellular microcompartments

The invention also covers methods for preparing cellular microcompartments to obtain the cellular microcompartment according to the invention. More particularly, the invention proposes creating cellular microcompartment containing pluripotent stem cells organized into cysts directly from pluripotent stem cells, or from differentiated cells which will be reprogrammed into pluripotent cells inside the hydrogel capsule during the formation of the microcompartments.

Any method for producing cellular microcompartments containing extracellular matrix and pluripotent stem cells inside a hydrogel capsule may be used for the implementation of the preparation method according to the invention. In particular, it is possible to prepare microcompartments by adapting the method and the microfluidic device described in Alessandri et al., 2016 ("A 3D printed microfluidic device for production of functionalized hydrogel microcapsules for culture and

differentiation of human Neuronal Stem Cells (hNSC)", Lab on a Chip, 2016, vol. 16, no. 9, pp. 1593–1604), in accordance with the steps described below.

In a first embodiment, the preparation method according to the invention comprises the steps of

- 5 (a) incubating human pluripotent stem cells in a culture medium containing a RHO/ROCK pathway inhibitor;
 - (b) mixing the pluripotent stem cells from step (a) with an extracellular matrix;
 - (c) encapsulating the mixture from step (b) in a hydrogel layer;
 - (d) culturing the capsules obtained in step (c) in a culture medium containing
10 a RHO/ROCK pathway inhibitor;
 - (e) rinsing the capsules from step (d) to remove the RHO/ROCK pathway inhibitor;
 - (f) culturing the capsules from step (e) for 3 to 20 days, preferentially for 5 to 10 days, until a cyst is obtained, and optionally recovering the obtained cellular microcompartments.
- 15 The incubation step (a) and culture step (d) in a medium containing one or more RHO/ROCK ("Rho-associated protein kinase") pathway inhibitors, such as Thiazovivin ($C_{15}H_{13}N_5OS$) and/or Y-27632 ($C_{14}H_{21}N_3O$), promote pluripotent stem cell survival and cell adhesion to the extracellular matrix during the formation of the hydrogel outer layer around said extracellular matrix. However, these steps should be
20 time-limited, so that RHO/ROCK pathway inhibitors do not prevent cysts from forming.

Thus, preferentially, the incubation of step (a) is carried out for a time included between a few minutes and a few hours, preferentially between 2 minutes and 2 hours, more preferably between 10 minutes and 1 hour.

- Likewise, preferentially, step (d) of culture is carried out for a time of between 2
25 and 48 hours, preferentially for a time of between 6 and 24 hours, more preferentially for a time of between 12 and 18 hours.

Step (e) is necessary to ensure that all traces of RHO/ROCK pathway inhibitors are removed. Step (e) is carried out by rinsing, and preferably a plurality of times, in successive culture media free from RHO/ROCK pathway inhibitors.

Advantageously, step (f) is carried out for a time sufficient to obtain a cellular
5 microcompartment wherein the layer of pluripotent cells and the lumen have a cumulative thickness equal to 10 to 95% of the radius of the microcompartment, i.e. for a time sufficient to allow the passage from two cells to around a thousand cells. Any culture medium suitable for culturing pluripotent stem cells, can be used, and in particular phosphate-buffered saline such as the “Rowell Park
10 Memorial Institute medium”.

In one embodiment, the method according to the invention comprises an intermediate step (a') consisting of dissociating the pluripotent stem cells from step (a) prior to step (b), preferably using an enzyme-free reagent. Advantageously, said reagent is inhibited or rinsed before the encapsulation step, notably by successive
15 rinses in a specific medium for pluripotent cells. For example, the reagent used is an iso-osmotic buffer containing EDTA or EGTA, such as ReLeSR®. Of course, it is also possible to use trypsin or an enzyme-containing reagent, but the survival rate of pluripotent cells at the end of this step may then be lower compared with the use of an enzyme-free reagent. In all cases, the rinsing step is necessary to remove all
20 traces of the reagent used for cell dissociation.

In one embodiment, at least one of the steps (a'), (b), (c), (d) or (e) is carried out at a temperature of between 0 and 8°C, preferably all the steps (a'), (b), (c), (d) and (e). Maintaining a temperature of around 4°C puts the cells' biological
25 processes into dormancy, including the transduction of signals from the external environment. This makes it possible to limit the phenomenon of cell death, which could be induced by cell detachment.

In another embodiment, the method for preparing a cellular microcompartment according to the invention comprises the steps of

(a) mixing differentiated human cells with extracellular matrix and with cell
30 reprogramming agents that do not permeate the hydrogel layer;

- (b) encapsulating the mixture from step (a) in a hydrogel layer;
- (c) culturing the capsules from step (b) for 10 to 40 days, and optionally recovering the cellular microcompartments obtained.

In another embodiment, the method for preparing a cellular microcompartment
5 according to the invention comprises the steps of

- (a) mixing differentiated human cells with extracellular matrix ;
- (b) encapsulating the mixture from step (a) in a hydrogel layer;
- (c) incubating the capsules from step (b) with cell reprogramming agents that permeate the hydrogel layer and culturing the capsules for 10 to 40 days,
10 optionally recovering the resulting cellular microcompartments.

For example, the differentiated cells used are fibroblasts.

A person skilled in the art knows how to reprogram a differentiated cell into a stem cell by reactivating the expression of genes associated with the embryonic stage by means of specific factors, designated in the present invention as
15 “reprogramming agents.” The methods described in the following may be cited as examples: Takahashi et al., 2006 (“Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors” Cell, 2006 Vol 126, pages 663–676), Ban et al., 2009 (“Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus
20 that does not integrate into the host genome” Proc Jpn Acad Ser B Phys Biol Sci. 2009; 85(8):348-62) and the international application WO2010/105311, the title of which is “Production of reprogrammed pluripotent cells”.

The reprogramming agents are advantageously co-encapsulated with the differentiated cells, so as to concentrate the product and promote the contact with all
25 of the cells. In the case of reprogramming agents permeating the hydrogel layer, it is possible to add said agents to the culture medium after the encapsulation step.

Reprogramming agents make it possible to impose a succession of phenotypic changes on the cells to the pluripotent stage. Advantageously, the step (a) of

- reprogramming is carried out using specific culture media, promoting these phenotypic changes. For example, the cells are cultured in a first medium comprising 10% of human or bovine serum, in an Eagle's minimum essential medium (DMEM) supplemented with a serine/threonine protein kinase receptor inhibitor (such as the product SB-431542 ($C_{22}H_{16}N_4O_3$)), one or more RHO/ROCK (RHO-associated protein kinase) pathway inhibitors, such as thiazovivin and/or Y-27632, fibroblast growth factors, such as FGF-2, ascorbic acid and antibiotics, such as Trichostatin A ($C_{17}H_{22}N_2O_3$). Then the culture medium is replaced with medium promoting the multiplication of the pluripotent cells, such as the mTeSR[®]1 medium.
- 10 Advantageously, the capsules resulting from step (b) each contain between 1 and 500 differentiated cells, preferably between 50 and 200.

- In one embodiment, at least one of the steps (a), (b), (c), or (d) is carried out at a temperature of between 0 and 4°C, preferably all the steps (a), (b), (c), and (d). Maintaining a temperature less than or equal to 4°C puts the cells' biological
- 15 processes into dormancy, including the transduction of signals from the external environment. This makes it possible to limit the phenomenon of cell death, which could be induced by cell detachment.

- The microcompartments obtained in step (d) can be sorted to isolate cellular microcompartments with the desired cyst shape. Such a sorting step can be
- 20 carried out continuously, so as to separate cellular microcompartments already presenting the desired cyst shape, from microcompartments still in the process of formation. Such a sorting step can be carried out by simple morphological analysis, without disturbing other microcompartments in which reprogramming is still in progress and/or cyst organization is not yet complete.

- 25 Generally speaking, the cellular microcompartments obtained according to the methods of the invention can then be frozen before any use. Indeed, the cyst shape favors cell survival within the microcompartment, and after thawing, the survival rate is over 80%. Advantageously, freezing is carried out using liquid nitrogen, enabling rapid vitrification of the microcompartments and limiting the risk of crystals forming
- 30 within the lipid membranes of the cells. Cell microcompartments can be suspended

in a freezing buffer to promote cell survival. It is possible, for example, to use freezing buffers that are conventionally used to freeze embryos.

The frozen cellular microcompartments can then be thawed as required.

Applications

- 5 The cellular microcompartments that form the subject of the present invention can be used for a wide range of applications. In fact, the cells they contain can be easily recovered by simple hydrolysis and/or dissolution of the hydrogel outer layer. In addition, pluripotent cells can be differentiated within the hydrogel capsule or after hydrolysis/dissolution of said hydrogel capsule, as required, so
- 10 as to obtain large quantities of cell lines of interest. Advantageously, the cells are differentiated into one or more cell types of interest within the microcompartment, i.e. before hydrolysis of the hydrogel outer layer.

Cellular microcompartments, and more precisely the cells they contain, can be used for research and development purposes, both in the form of a 3D cellular

15 network and more conventionally in 2D culture. They can also be used for therapeutic purposes, such as cell therapy and tissue engineering.

Examples

Example 1: Protocol for obtaining cellular microcompartments from human cells induced to pluripotency

20 Solutions used

Solution 1, based on DMEMF12 medium supplemented with 2 μ M Thiazovivin

Solution 2, magnesium- and calcium-free PBS supplemented with 1 μ M 2 μ M Thiazovivin

Solution 3, non-enzymatic cell detachment buffer: RelesR[™] supplemented with

25 2 μ M Thiazovivin.

Solution 4, pluripotent stem cell culture medium: MTeSR1[™] hES/hIPS cell medium STEMCELL[™]).

Solution 4+, Solution 4 supplemented with 2 μ M Thiazovivin.

Solution 5 Matrigel™.

Solution 6, 300mM sorbitol with 2 μ M Thiazovivin.

Cellular solution

- 5 A Petri dish of human IPS cells (obtained from Primary Dermal Fibroblast; Normal, Human, Adult ATCC® PCS-201-012™ and CytoTune™-iPS 2.0 Sendai Reprogramming Kit (ref A16517) using the technology presented in Example 2) of 25cm² at 90% confluence is used afterward to match the recommended volumes. All subsequent steps are carried out at 4°C until the hydrogel shell
- 10 cross-links in the calcium bath.

Step 1: Rinse the cells with solution 1. Wait 10 minutes to 1 hour.

Step 2: Rinse twice with 4 mL of solution 2.

Step 3: Gently suction the solution.

Step 4: Incubate the cells with 4 mL of solution 3 for 5-10 minutes.

- 15 Step 5: Detach cells with 2 mL of 4+ solution using a wide cone pipette to reduce shear stress.

Step 6: Centrifuge the cell suspension at 360 g for 5 minutes.

Step 7: Suction the supernatant.

Step 8: Resuspend with 0.5 mL of solution 4+.

- 20 Step 9: Centrifuge again at 360 g and suction the supernatant.

Step 10: Resuspend the cell pellet in 70 μ L of solution 5 and 100 μ L of solution 6 (the volume of the pellet should be 30 μ L). The cell solution is ready.

Encapsulation

The encapsulation device is prepared as described in Alessandri et al., 2016 ("A 3D printed microfluidic device for production of functionalized hydrogel microcapsules for culture and differentiation of human Neuronal Stem Cells (hNSC)", Lab on a Chip, 2016, vol. 16, no. 9, pp. 1593-1604).

To summarize, the various parts of the device are sterilized (in an autoclave); the three required solutions are loaded onto three syringe pumps: i) alginate solution (PRONOVA®SLG100 at 2% by weight in distilled water), ii) intermediate solution (sorbitol at 300mM), iii) cell solution (prepared in the previous step). The three solutions are coinjected concentrically using a microfluidic injector to form a jet that splits into drops with the alginate solution as the outer layer and the cell solution as the core. These drops are collected in a calcium bath (at 100mM), which stiffens the alginate solution to form the shell.

To improve the monodispersity of the cellular microcompartments, the alginate has been charged is charged with a +2KV direct current. A ring at ground, 2 cm in diameter, is arranged 500µm away from the tip in the plane perpendicular to the axis of the jet emerging from the microfluidic injector in order to generate the electric field.

Note that under these encapsulation conditions, the Matrigel® layer forms spontaneously.

20 Treatment after encapsulation

Step 1: Capsules are recovered using a 40 µm cell sieve, then rinsed with solution 1 and stored in a 75 cm² flask with 2 0mL of solution 4+.

Step 2: The flask is kept for 12h in an incubator at 37°C and 5% CO₂.

Step 3: Change the medium to solution 4 to allow cysts to form.

25 Step 4: After 24 to 72 hours, cysts of a few dozen cells form in the capsules. Cell microcompartments mature after 5 to 10 days.

Example 2: Protocol for obtaining cellular microcompartments from human fibroblasts

Solutions used

Solution 1, DMEMF12 medium base

Solution 2, PBS magnesium-free calcium-free supplemented

Solution 3, trypsin EDTA cell detachment buffer

- 5 Solution 4, fibroblast culture medium: 10% human serum in DMEM medium base

Solution 4+, Solution 4 supplemented with 2 μ M Thiazovivin.

Solution 5 Matrigel™.

Solution 6, 300mM sorbitol with 2 μ M Thiazovivin.

Cellular solution

- 10 A 25cm² Petri dish of Primary Dermal Fibroblast; Normal, Human, Adult (ATCC® PCS-201-012®) at low confluence density is then used to match the recommended volumes (1 to 2 million cells). All subsequent steps are carried out at 4°C until the shell cross-links in the calcium bath.

Step 1: Rinse the cells with solution 2.

- 15 Step 2: Gently suction the solution.

Step 3: Incubate the cells with 4 mL of solution 3 for 5-10 minutes.

Step 4: Detach cells with 2 mL of 4+ solution using a wide cone pipette to reduce shear stress.

Step 6: Centrifuge the cell suspension at 360 g for 5 minutes.

- 20 Step 7: Suction the supernatant.

Step 8: Resuspend with 0.5 mL of solution 4+.

Step 9: Centrifuge again at 360 g and suction the supernatant.

Step 10: Resuspend the cell pellet in 90 μL of solution 5 and 100 μL of solution 6 (the volume of the pellet should be 10 μL).

Step 11: Add 1/10 of the contents of the "CytoTune® -IPS 2.0 Sendai Reprogramming Kit" (containing a reprogramming virus) intended for a 6-well
5 plate. The cell solution is ready.

Encapsulation

Encapsulation is carried out according to the protocol in Example 1.

Treatment after encapsulation

Step 1: Capsules are recovered using a 40 μm cell sieve, then rinsed with
10 solution 1 and stored in a 75 cm^2 flask with 20 mL of solution 4+.

Step 2: The flask is kept for 24h in an incubator at 37°C and 5% CO_2 .

Step 3: Change the medium every day. Each capsule contains 1 to 10 fibroblasts when the capsules are formed. The reprogramming virus has a transformation efficiency of around 0.2%. Most capsules will therefore contain few or no
15 reprogrammed cells. Cysts begin to form after 15 to 40 days. The fibroblasts are elongated and do not form cysts. All the cysts that form are made up of IPS.

PATENTKRAV

1. Cellulært mikrokompartment omfattende, successivt og organiseret om et hulrum:
 - i det mindste et lag af menneskelige pluripotente celler;
 - 5 - et extracellulært matrixlag;
 - et ydre hydrogellag,hvor mikrokompartmentet er lukket.
2. Cellulært mikrokompartment ifølge krav 1, hvor det ydre lag omfatter alginat.
- 10 3. Cellulært mikrokompartment ifølge et hvilket som helst af de foregående krav, hvor mikrokompartmentet har kugleform eller en aflang form.
4. Cellulært mikrokompartment ifølge et hvilket som helst af de foregående krav, hvor mikrokompartmentet har en diameter eller en mindre dimension på mellem 10 μm og 1 mm, fortrinsvis mellem 50 μm og 500 μm , mere fortrinsvis
15 mindre end 500 μm , endnu mere fortrinsvis mindre end 400 μm .
5. Cellulært mikrokompartment ifølge et hvilket som helst af de foregående krav, hvor celletætheden er mellem en og flere tusinde celler, fortrinsvis 50 til 1000 celler pr. mikrokompartment.
6. Fremgangsmåde til fremstilling af et cellulært mikrokompartment ifølge et
20 hvilket som helst af krav 1 til 5, omfattende følgende trin:
 - (a) inkubation af menneskelige pluripotente stamceller i et dyrkningsmedium indeholdende en RHO/ROCK pathway-inhibitor;
 - (b) blanding af de pluripotente stamceller fra trin (a) med en extracellulær matrix;
 - (c) indkapsling af blandingen fra trin (b) i et hydrogellag;
 - 25 (d) dyrkning af kapslerne tilvejebragt i trin (c) i et dyrkningsmedium indeholdende en RHO/ROCK pathway-inhibitor;

(e) skylning af kapslerne fra trin (d) for at fjerne RHO/ROCK pathway-inhibitoren;

(f) dyrkning af kapslerne fra trin (e) i 3 til 20 dage, fortrinsvis i 5 til 10 dage, og eventuelt genvinding af de tilvejebragte cellulære mikrokompartmente.

7. Fremgangsmåde til fremstilling af et mikrokompartment ifølge krav 6,

5 hvilken fremgangsmåde omfatter et mellemliggende trin bestående i

(a') dissociering af de pluripotente stamceller fra trin (a) inden trin (b), fortrinsvis ved hjælp af en enzymfri reagens.

8. Fremgangsmåde til fremstilling af et cellulært mikrokompartment ifølge et hvilket som helst af krav 1 til 6, omfattende følgende trin:

10 (a) blanding af menneskelige differentierede celler med en extracellulær matrix og celleomprogrammeringsstoffer;

(b) indkapsling af blandingen fra trin (a) i et hydrogellag;

(c) dyrkning af kapslerne fra trin (b) i 10 til 40 dage og eventuelt genvinding af de tilvejebragte cellulære mikrokompartmente.

15 9. Fremgangsmåde til fremstilling af et cellulært mikrokompartment ifølge krav 8, hvor hver kapsel fra trin (b) indeholder mellem 1 og 500 differentierede celler.

10. Fremgangsmåde til fremstilling af et cellulært mikrokompartment ifølge et hvilket som helst af krav 6 til 10, hvilken fremgangsmåde omfatter et yderligere trin bestående i frysning af de cellulære mikrokompartmente, der er tilvejebragt i trin (f)

20 ifølge krav 6 eller i trin (c) ifølge krav 8.

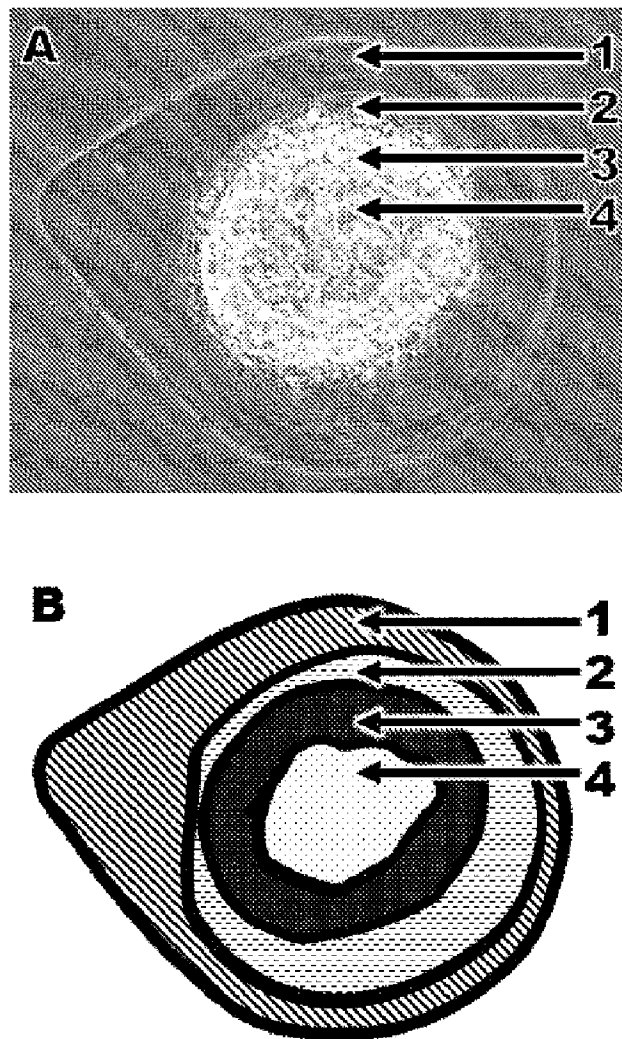


FIGURE 1