BIOPRINTING PROTOCOL FOR HUMAN EAR WITH CELLS

Overview: This protocol is a specific way to a human ear from an MRI scan using CELLINK bioink and human primary nasal chondrocytes (hNC).

Materials:
Human ear MRI Scan at a scale of 35% of original size[1]
Slic3r Software (v1.2.9)
CELLINK bioink[2,3]
Human primary nasal chondrocytes (hNC), at a concentration of $20x10^6$ cells/ml
Cell Culture Medium, as described below
Differentiation Medium, as described below
CELLMIXER Kit
INKREDIBLE 3D Bioprinter by CELLINK
Conical tip, 250µm ID
$CaCl_2$ Crosslinking Solution

Protocol:

1. The first step is to upload the human ear MRI to the Slic3r software to create an STL file. Using Slic3r (v1.2.9), convert the 3D model to a bioprinting protocol and toolpath with the following parameters:
    - Layer height = 0.40mm
    - External perimeters extrusion width = 0.45mm
    - Perimeters = 1
    - Infill density = 30%
    - Infill Pattern = Rectilinear
    - Printing speed, F = 600mm/min

    Upload the bioprinting protocol with the following name:
    "HumanEar_Scale35_LH04_Infill30_F600.gcode"

2. The human primary nasal chondrocytes must be prepared at a concentration of $20x10^6$ cell/ml. First, prepare a cell suspension of $72x10^6$ hNCs in a volume of 600µL were mixed

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with 3mL of CELLINK bioink using the CELLMIXER to obtain a final concentration of $20x10^6$ cells/ml.

Please watch the video in this link for a detailed illustration on how to do the mixing process: **https://www.youtube.com/watch?v=CmSYL1-oltI .** This gives a final ratio of 10:1 (bioink to cell suspension).

3. The following materials are used in the culture medium: DMEM/Ham's F-12 (1:1, Biochrom), supplemented with 10% fetal bovine serum (Biochrom) and 1% penicillin–streptomycin

   The following materials are used in the differentiation medium: StemMACS ChondroDiff media (Miltenyi Biotec GmbH) supplemented with 1% penicillin–streptomycin.

4. The following bioprinting parameters can be used with the INKREDIBLE 3D Bioprinter by CELLINK using the pneumatic-driven micro-extrusion technology.

   - Conical tip, 250µm ID (25 G)
   - Pressure: 15-17 kPa
   - Printing speed: 600 mm/min
   - Printhead temperature: Room temperature (22°C)
   - Printbed temperature: Room temperature (22°C)

5. After the bioprinting process, the cell-laden ear constructs were crosslinked by submerging in an ionic solution of 100mM $CaCl_2$ for 10 minutes. The constructs were then rinsed and incubated in culture medium in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity).

6. Bioprinting metrics
   a. Time for bioprinting: 5 minutes and 51 seconds per construct
   b. Volume of bioink per construct: 1.2 mL

7. After the bioprinting process, cells were in 3D culture in vitro for 28 days.

8. The stability of constructs in vitro using macroscopic evaluation showed no change in shape after 7 days of *in vitro* culture.

G-codes:
HumanEar_Scale35_LH04_Infill30_F600.gcode

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Further Information:
human ear.stl

References:

1. Nimeskern, L. *et al.* Mechanical evaluation of bacterial nanocellulose as an implant material for ear cartilage replacement. *J Mech Behav Biomed* **22**, 12-21, dx.doi.org/10.1016/j.jmbbm.2013.03.005 (2013).
2. 3D bioprinting of human chondrocyte-laden nanocellulose hydrogels for patient-specific auricular cartilage regeneration. Héctor Martínez Ávila, Silke Schwarz, Nicole Rotter, Paul Gatenholm. *Bioprinting* **2016**, Volumes 1–2, 22-35.
3. 3D Bioprinting Human Chondrocytes with Nanocellulose–Alginate Bioink for Cartilage Tissue Engineering Applications. Kajsa Markstedt, Athanasios Mantas, Ivan Tournier, Héctor Martínez Ávila, Daniel Hägg, and Paul Gatenholm. *Biomacromolecules* **2015** *16* (5), 1489-1496. dx.doi.org/10.1021/acs.biomac.5b00188

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