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Design of 3-D adipospheres for quantitative metabolic study

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Abstract

Quantitative assessment of adipose mitochondrial activity is critical for better understanding of adipose tissue function in obesity and diabetes. While the two-dimensional (2-D) tissue culture method has been sufficient to discover key molecules that regulate adipocyte differentiation and function, the method is insufficient to determine the role of extracellular matrix (ECM) molecules and their modifiers, such as matrix metalloproteinases (MMPs), in regulating adipocyte function in three-dimensional (3-D) *in vivo*-like microenvironments. By using a 3-D hanging drop tissue culture system, we are able to produce scalable 3-D adipospheres that are suitable for quantitative mitochondrial study in 3-D microenvironment.

1. Introduction

In the adipose tissue, a group of adipocytes are clustered to form a grape-like structure that is enmeshed with collagen fibers (Chun et al. 2006; Khan et al. 2009). Within this three-dimensional (3-D) ECM scaffold, vascular and autonomic nervous system are intertwined to regulate the metabolic function of the adipose tissue (Diculescu and Stoica 1970). Vascular system provides nutrition and oxygen necessary for adipose tissue function, whereas autonomic nervous system regulates the lipolysis of adipocytes. Moreover, preadipocytes within adipose tissues may regulate the function of the adipose tissue through *de novo* adipogenesis as well as the interaction with adipocytes in paracrine and juxtacrine manners. Adipocyte differentiation and function in 3-D environment significantly differ from those in 2-D (Chun et al. 2006); therefore, assessing the metabolic function of the adipose tissue in a 3-D configuration is a critical step forward to better understand the 3-D adipose tissue metabolism. Herein, we describe a high-throughput tissue culture method to prepare 3-D adipospheres (Moraes et al. 2013; Tung et al. 2011) and measure their mitochondrial respirations. Through this approach, we are able to control the quantity of adipocytes per a miniature adipose tissues as well as ECM components depending on the experimental purposes. The physiological and pathological (fibrotic) 3-D adipospheres are ideal platforms to determine adipose tissue function in the *in vivo*-like environment. 3-D adipospheres can

serve as a functional adipose unit useful for the better understanding of 3-D adipose tissue function.

2. Materials

2.1 Hanging drop culture of preadipocyte spheroids

1. Perfecta3D Hanging Drop Plates from 3D Biomatrix, Ann Arbor, MI, USA.
2. Sterilized water
3. **Hanging drop culture medium:** high-glucose DMEM containing (catalog no. 11965-092, Gibco, Thermo Fisher Scientific, Waltham, MA), 10% fetal bovine serum (FBS, catalog no. 16000-044, Gibco), 2 mM glutamine, 100 U/ml of penicillin, and 100 U/ml of streptomycin.
4. 1.2% Methocel A4M (Sigma, St. Louis, MO, USA), dissolved into hanging drop culture media.
5. Multichannel pipette
6. Water reservoir
7. 3T3L1 cells (ATCC, Manassas, VA) or any other preadipocytes.

2.2. Induction of adipocyte differentiation

1. **Adipocyte differentiation medium:** high-glucose DMEM, 10% FBS, 2 mM glutamine, 100 U/ml of penicillin, 100 U/ml of streptomycin, 250 nM dexamethasone, 10 μ M troglitazone, 10 nM T3, and 1 μ g/ml insulin.
2. **Insulin medium,** DMEM, 10% FBS, 2 mM glutamine, 100 U/ml of penicillin, 100 U/ml of streptomycin, and 1 μ g/ml insulin.

2.3. Mitochondrial respiration assay

1. XFe96 Spheroid FluxPak (Seahorse Biosciences, North Billerica, MA, USA).
2. Corning Costar Ultra-Low attachment multiwell plate (Corning, Corning, NY, USA).
3. More than 50 ml of **Mitochondrial Assay Medium** containing XF Assay Medium Modified DMEM (Seahorse Biosciences), 25 mM glucose, and 2 mM sodium pyruvate adjusted to pH 7.3–7.4 (see Note 1).
4. 3 ml of 2 μ M oligomycin (Sigma) in mitochondrial assay medium.
5. 3 ml of 0.5 μ M carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, from Sigma) in assay medium.
6. 3 ml of 1 μ M antimycin A and 1 μ M rotenone (all from Sigma) in assay medium.

¹Substrates and their optimal concentrations to be used in mitochondrial assays may vary depending on the cell type and the metabolic pathways of researcher's interest.

7. Incubator at 37 °C supplying no CO₂.
8. XFe96 Extracellular flux analyzer with a Spheroid microplate-compatible thermal tray from Seahorse Biosciences.

3. Methods

3.1. Spheroid formation and adipocyte differentiation

1. Supply sterilized water into designated hydration reservoir in a bottom plate or in a bottom tray (see ^{Note 2}).
2. Prepare cell suspension containing appropriate number of cells in 25 µl hanging drop culture medium per drop (Fig. 1, see ^{Note 3})(Tung et al. 2011). The addition of Methocel at 0.24% final concentration promotes the formation of a single spheroid per hanging drop instead of multiple small satellite spheroids.
3. Transfer cell suspension into a reservoir trough and dispense 25 µl each into a hanging drop plate using a multichannel pipette.
4. Hanging drop plate can be detached from the bottom reservoir and placed directly on a microscope stage during observation. Confirm that a single spheroid is formed in a well after one day. At 2 days post-seeding change media to adipocyte differentiation media. To ensure complete medium change replace half of droplet volume (approx. 9–10 µL) with fresh medium. Repeat 7 times to achieve <1% of residual medium in hanging drop. (see ^{Note 4}).
5. Medium is changed every other day by replacing 9–10 µl three times. Note the size of droplet to determine if more or less medium is needed per change cycle.
6. Four days after treatment with adipocyte differentiation medium, change media to insulin media.
7. Culture two more days to complete differentiation (Fig. 2).

3.2. Mitochondrial activity assay

1. A day before assay, prepare a sensor cartridge by adding 200 µl of XF calibrant into each well of a 96-well bottom, which is included in XFe96 Spheroid FluxPak. Incubate the prepared cartridge in an incubator at 37 °C overnight (see ^{Note 5}).
2. Take pictures of microscope to measure spheroid diameter under inverted microscope.

²To avoid water flowing out by tilting, hot 1% agarose is poured to solidify after cooling or wet Kimwipes are used.

³Appropriate volume for single hanging drop culture is 20 – 30 µl. Spheroids tend to adhere to the side of the plate when the culture volume is less than 15 µl and fall down to the bottom when more than 35 µl.

⁴Medium change can be automated by using a liquid handler CyBi-Well together with an Adapter Standard 384.SQW6 and CyBi-TipTray 96 (all from CyBio, Jena, Germany).

⁵The sensor cartridge will be ready to use after 4-hour incubation, but overnight incubation is recommended to stable data.

3. Add mitochondrial assay media into wells of an Ultra-Low attachment multiwell plate. Place the plate below the drop plate with hanging spheroids. Let a hanging spheroid drop down by the addition of 30 μ l assay medium (see Note 6 and 7).
4. Wash spheroids a few times with mitochondria assay media (see Note 8).
5. Add 165 μ l Assay medium into a XFe96 spheroid microplate in a XFe96 spheroid FluxPak.
6. Cut a tip and aspirate 10 μ l with a spheroid. Transfer spheroids into the XFe96 spheroid microplate (see Note 9). Do not transfer spheroids into wells at corners, instead, add only Assay medium.
7. Incubate the XFe96 spheroid microplate in the incubator at 37 °C for one hour.
8. Dispense 25 μ l of oligomycin, FCCP, and antimycin A/rotenone solution into injection ports A, B, and C in the sensor cartridge (see Note 10).
9. Launch WAVE program in XFe96 Extracellular flux analyzer system. Fill out plate design according to the transferred spheroids into the XFe96 spheroid microplate. Well volume is set to 175 μ l.
10. Set measurement cycles after 12 minutes of equilibration. For all of the cycles, set 3 minutes for mixing and measurement. There are 5 cycles before injection as basal activity level. After injection of oligomycin from port A, run 12 cycles. Then inject FCCP from port B followed by 5 cycles. After injection of antimycin A and rotenone, 7 cycles last to end of the measurement (Fig. 3).

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⁶As an alternative way, spheroids are collected at once to spin down by using Perfecta3D Spheroid Transfer Tool from 3D Biomatrix. Centrifugation speed may vary depending on cell type and spheroid size. It is also possible to pick up a spheroid from the plate by manually aspirating with a cut tip.

⁷If standard cell culture plates are used to collect spheroids, they may adhere to the bottom of the plates quickly. If spheroids are collected into a microtube, they may adhere each other.

⁸It is important to reduce bicarbonate ion contained in standard DMEM by washing with mitochondrial assay medium because pH is measured during assay to calculate Extracellular acidification (ECAR). XF Assay Medium Modified DMEM doesn't contain sodium bicarbonate to buffer pH.

⁹A few spheroids may not stay in the center of a well during a mitochondrial assay, which is observed as a sudden and artificial decrease of a measured value. To avoid this problem, wells of XFe96 spheroid microplates can be pre-coated with an adhesive to hold spheroids onto the center. When poly-D-lysine is used for coating, 30 μ l of 0.1 mg/ml poly-D-lysine solution is added into each well to incubate for 20 minutes. After aspiration, wells are washed twice with sterilized water followed by complete drying up.

¹⁰The concentrations of these antibiotics are optimized for specific types of cells.

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Fig. 1. 3-D hanging drop tissue culture. A. Schematic view of 384-well hanging drop culture system. B. Overview of the 384-well hanging drop culture plate. C. The procedures for spheroid formation. Reprinted from Tung et al., 2011.

Fig. 2.

Cell number- and differentiation-dependent regulation of spheroid size. A. a spheroid of preadipocytes (20K cells) without adipogenic mix (left), adiposphere with adipogenesis. Scale = 200 μm. B. Increased size of adipospheres with increased number of preadipocytes used. Scale = 200 μm. C. Linear relationship between the diameter of adipospheres and the initial number of preadipocytes used. Mean ±SEM. N=6

Fig. 3.

Differentiation- and cell number-dependent oxygen consumption rate (OCR). A. Differentiated adipocytes (closed circles) demonstrate a robust OCR compared to preadipocytes (open circles). With the use of a proton ionophore, FCCP, maximal OCRs under uncoupled states are observed. Oligomycin, an inhibitor of complex V, ATP synthase blocks ATP-synthesizing mitochondrial respiration. The use of both Antimycin A, a complex III inhibitor and rotenone, a complex I inhibitor, completely shuts down mitochondrial respiration.