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Single Cell Densitometry and Weightlessness Culture of Mesenchymal Stem Cells Using Magnetic Levitation

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Abstract

Magnetic levitation methodology enables density-based separation of microparticles/cells and sustains cell culture in different media. Levitation process can be accomplished via negative magnetophoresis (diamagnetophoresis), where the applied magnetic force compensates gravitational acceleration and the density of the diamagnetic object (e.g., cell) determines its levitation height. Here we describe a portable, sensitive, and cost-effective technology that uses the principles of magnetic levitation to measure single cell density and cell culture under desired conditions.

Keywords Magnetic levitation, Single cell density, Simulated microgravity, Stem cells, Real-time monitoring

1 Introduction

Single cell density is an important cellular phenotype that can indicate parameters such as cell state or differentiation [1, 2]. It is possible to measure cell density by using different methodologies including density-gradient centrifugation, suspended microchannel resonator (SMR) [3], and optically induced electrokinetics (OEK) [4]. However, these systems either allow the density measurement of a bulk cell population, or their applications are considerably limited due to the expensive and complex operation requirements [1]. Magnetic levitation through negative magnetophoresis is a novel and simple technology to detect single cell density and to perform density-based cell separation. This method allows cells suspended in a magnetic medium (paramagnetic salt solution or ferrofluid) to be directed toward the lower magnetic field induction site and to be positioned at different levels of the magnetic field gradient depending on their densities. Magnetic levitation technology, which was originally designed for the manipulation of nonliving materials [5–8], afterward was applied to biological com-

ponents for separation and density measurements. Many types of cells, especially stem cells, cancer cells, and circulating cells, have been successfully separated using magnetic levitation [1, 9, 10].

The magnetic levitation technology can also be extended to culture cells in weightlessness with a suitable experimental design. Weightlessness research is quite important for space biology applications; however, common methodologies are limited to in vivo tissue level studies that can be expensive and challenging [11, 12]. Cell-based weightlessness techniques also require additional mechanical devices (such as rotating wall vessel or random positioning machine) that consequentially generate external shear forces during their operation [13, 14]. Besides, these devices do not allow real-time monitoring. Magnetic levitation presents an alternative solution for cell-based weightlessness research, because not only it allows real-time monitoring, but also it can inherently cancel individual weight of the cells without generating any other force [15]. Therefore, cell culture with magnetic levitation provides a useful tool to culture cells under simulated weightlessness [16]. It is also possible to code individual cells into functional three-dimensional (3D) architectures [17].

The technology is moving toward implementing in point-of-care settings [18, 19] and simulating microgravity for space biology applications [16, 20]. Here, we comprehensively describe a standard protocol for label-free magnetic levitation of stem cells, in which separation from a heterogenous population [15] and mechanical manipulation [21] may be required based on specific applications.

2 Materials

2.1 Microfluidic Setup

1. High-grade (N52) neodymium (NdFeB) magnets (50 mm length, 2 mm width, and 5 mm height) magnetized through their heights.
2. Micro-capillary channel (1 mm × 1 mm square cross section, 50-mm length).
3. Mirrors.

2.2 Magnetic Levitation

1. Cells of interest.
2. Cell culture medium specific to the cell type.
3. Fetal bovine serum (FBS).
4. Hemocytometer or cell counter.
5. Conical tubes, 15 mL.
6. Microcentrifuge tubes, 0.5 mL.
7. Pipettor, 10 μL and 200 μL.

8. Pipette tip, 10 and 200 μL.
9. Gd^{3+}-based contrast agent (Gadavist®, Bayer).
10. Capillary tube sealant (Critoseal).
11. Petri dish.
12. Polymeric microbeads with known densities.
13. Phosphate-buffered saline (PBS).
14. Tween 20.
15. Laminar flow cabinet.
16. Incubator (37 °C, 5% CO_2).
17. Inverted microscope.
18. An image processing software (ImageJ Fiji software).

3 Methods

Collect cells from the culture just before magnetic levitation procedures. Carry out all procedures at room temperature.

3.1 Microfluidic Setup

1. Position NdFeB magnets at 1.5 mm distance with same poles facing each other (*see* **Note 1**).
2. Place the micro-capillary channel as levitation chamber between two magnets.
3. Place mirrors on both sides of the capillary at 45° for real-time inverted microscope imaging of levitation chamber (*see* **Note 2**) (Fig. 1).

3.2 Sample Preparation

1. Culture cells using standard cell culture procedures and cell-type-specific supplies (*see* **Notes 3** and **4**).
2. For adherent cells, detach cells from the culture surface using the detachment protocol for the specific cell type, and transfer the cell suspension into a centrifuge tube.
3. For cells that grow in suspension, transfer the cell suspension into a centrifuge tube.
4. Centrifuge cells down at room temperature, remove medium, and resuspend in an appropriate solution before counting (*see* **Note 5**).
5. Count the cell number in suspension using either a cell counter or a hemocytometer.
6. Prepare the cell suspension at desired final cell concentration in the solution selected for levitation (*see* **Notes 6** and **7**).

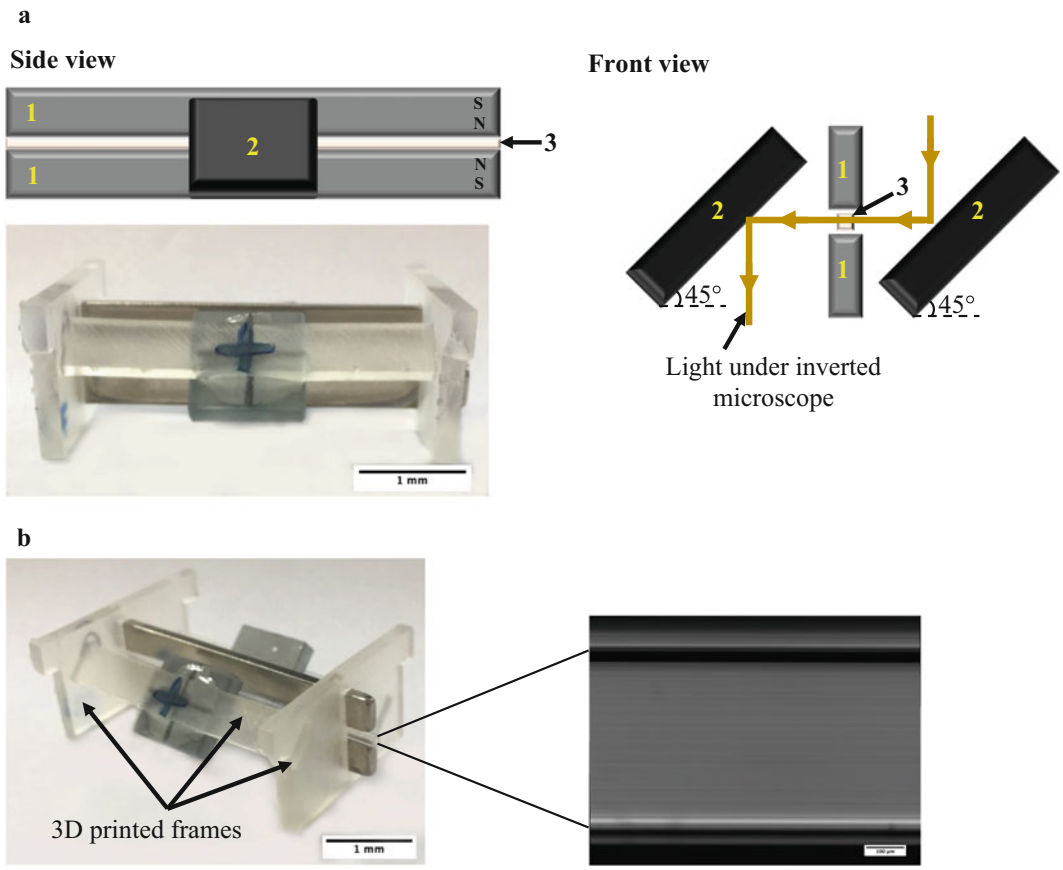

Fig. 1 Magnetic levitation setup. (**a**) Side and front view of the setup: (1) magnets, (2) mirrors, and (3) capillary channel. Scale bar, 1 mm. (**b**) Perspective view of the setup (scale bar, 1 mm) and micrograph of an empty levitation chamber (capillary channel) under an inverted microscope. The components for this specific magnetic levitation setup are held together with 3D-printed frames. (Scale bar, 100 μm)

3.3 Magnetic Levitation

1. Clean surfaces of the capillary channel and mirrors using a napkin moistened with ethanol (70%) before operation to get a clear image (*see* **Note 8**).
2. Add gadolinium (Gd^{3+})-based agent (gadobutrol, Gadavist®, Bayer) at the desired final concentration to the cell suspension to render the suspension paramagnetic just before levitation (*see* **Notes 9–12**) (Fig. 2).
3. Load the paramagnetic cell suspension into the capillary channel.
4. Seal the capillary channel with Critoseal, and insert the channel in the slot between the magnets.
5. For single cell level analysis, wait for cells to reach equilibrium height with real-time observation (*see* Subheading 3.4) (*see* **Note 13**).

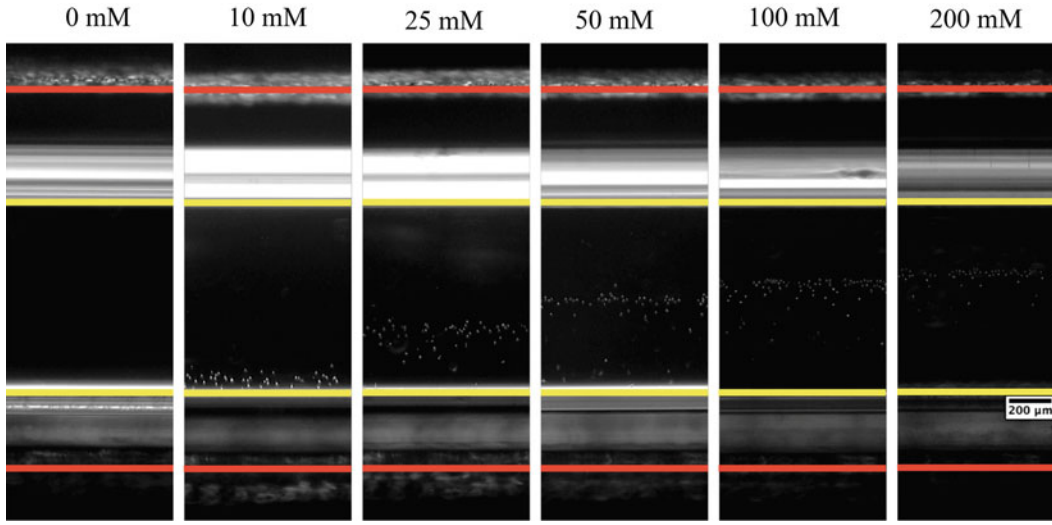

Fig. 2 Micrographs of levitated D1 ORL UVA cells (mouse bone marrow cell line) in the cell culture medium containing gadobutrol at variable concentrations (0, 10, 25, 50, 100, and 200 mM) after cells reached equilibrium position (~10 min). Red lines show top level of the bottom magnet and bottom level of the top magnet, and yellow lines show upper and lower levels of the inside of the capillary. Scale bar, 200 μm

6. For culture of cells with magnetic levitation, enclose the cells in the magnetic levitation setup in a petri dish with sterile water (~5 mL) to prevent excessive evaporation. Then, maintain the magnetic levitation culture in an incubator (37 °C, 5% CO_2).

3.4 Imaging and Analysis

1. Place the magnetic levitation setup on the microscope specimen stage using an adaptor part to hold the magnetic levitation setup close to the objective.
2. Focus the capillary by bringing the objective closer to the magnetic levitation setup using adjustment knob (*see* **Note 14**).
3. Adjust condenser height, optical element arrangement engaged in the light path and exposure time to obtain a clear image (*see* **Notes 15** and **16**).
4. Image the same area separately by focusing cells and magnets to get images in which both are fully focused.
5. Open images of the same area focusing cells and magnets in ImageJ Fiji software.
6. Select the area between the two magnets by rectangular selection tool on the image that the magnets are focused on, apply this interested area to the image of focused cells in the same area, and crop the image.
7. Use the command "subtract background" to remove potentially uneven backgrounds.

8. Set the threshold to properly represent cells as analyzable particles (*see* **Note 17**).
9. Set measurements including at least area and center of mass.
10. Select the area where all cells are located by rectangular selection tool, and analyze particles (*see* **Notes 18** and **19**).
11. Record the sizes of particles and distances between mass centers of particles and the top surface of the bottom magnet in the analysis window (*see* **Note 20**).

3.5 Calibration of Setup

In order to use magnetic levitation for measuring the density of cells or 3D structures, the system needs to be calibrated using beads with known densities (*see* **Note 21**).

1. Prepare 200 μL PBS-Tween solution by diluting 2% (volume/volume) Tween 20 in PBS.
2. Prepare stock solutions of each microbead: take the microbeads with a pipette tip, and suspend in PBS-Tween solution.
3. Count the beads in suspension using a hemocytometer.
4. Prepare bead suspensions in the solution used for cell levitation at a final concentration between 10^4 and 10^5 beads/mL (at least 50 μL final volume including gadobutrol for each magnetic levitation operation).
5. Add gadobutrol to the bead suspension at a final concentration which the cells were levitated. Then perform the magnetic levitation of beads (*see* Subheading 3.3).
6. Image levitation of beads, and analyze their levitation heights (distance between the center of masses and the top surface of the bottom magnet) (*see* Subheading 3.4).
7. Plot the graph showing levitation height versus density of beads, and perform linear regression over the data to obtain equation providing the density corresponding to the measured levitation height.
8. Convert the measured levitation heights to density using the resulting equation.

4 Notes

1. The magnetic field distribution can be altered by changing the distance between the magnets, and the expected field distribution can be simulated using finite element method. For in-depth theoretical representation and finite element solutions, please refer to [9, 15, 16] for the magnetic field distributions and [1] for expected cell localizations.

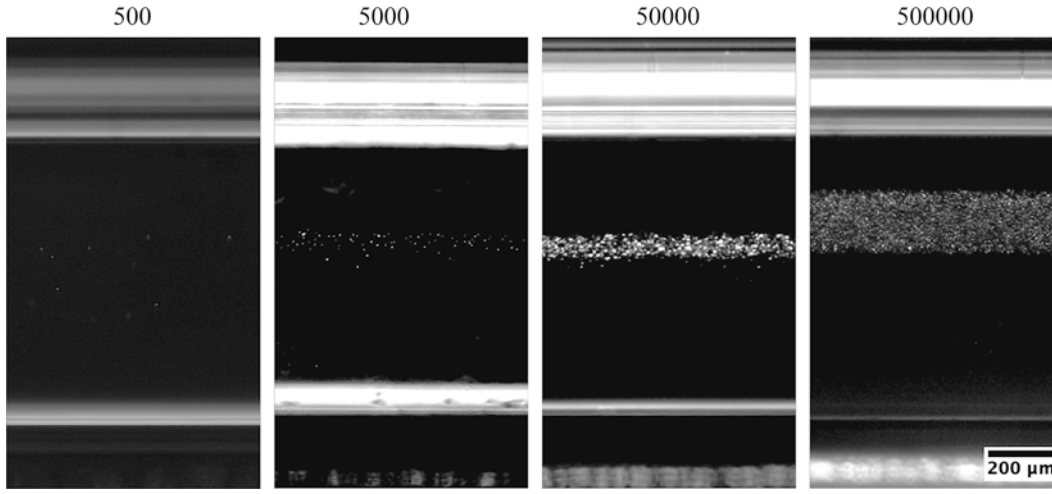

Fig. 3 Micrographs of levitated D1 ORL UVA cells at different cell numbers (total 500, 5000, 50000, and 500000 cells) after 10 min of levitation in the medium containing 100 mM gadobutrol. Scale bar, 200 µm

2. The components of the magnetic levitation setup can be held together using 3D-printed [16] or laser-cut [9, 22] connecting pieces.
3. This protocol is prepared based on stem cells of bone marrow origin with adipogenic and osteogenic potential [23], for other stem cell types apply appropriate stem cell culture techniques based on the current hypothesis.
4. If using frozen cells, follow step 4 after rapid thawing (<1 min).
5. For live cell levitation, different solutions can be used, such as cell-specific medium [16] or FBS [9], depending on the purpose. The use of culture medium is needed to culture cells under the condition of magnetic levitation, whereas FBS may be more suitable for levitation of cells with different densities at distinct levitation levels and separation of them.
6. The cell concentration to be used should be chosen by considering the purpose as it will change the order of the cells within the capillary (Fig. 3). For instance, cell concentration of 10^5 cells/mL or less is found to be appropriate to individually visualize relatively large cells such as adipocytes and to measure their density at a single cell level [1]; in cell culture with magnetic levitation, this number can be increased to form larger 3D strings rather than spheroids [16, 22].
7. Since the capillary has a volume of about 50 µL, the cell suspension volume prepared for a single magnetic levitation operation should be prepared to be at least 50 µL after adding gadobutrol.

8. To use magnetic levitation setup for cell culture, "magnetic levitation" steps following the "sample preparation" steps should be carried out under sterile conditions. In accordance, sterilize components of magnetic levitation setup by UV exposure (30 min) before this step.
9. Lanthanide ions such as Gd^{3+} have approximately the same size as Ca^{2+} and can cause competitive inhibition of cellular processes requiring Ca^{2+} and therefore lead to cytotoxicity [24]. Chelation by a ligand mitigates direct toxicity of free Gd^{3+}. Commercially available chelate forms of Gd^{3+} can provide a proper biocompatibility. Gadobutrol (Gadavist®) is a suitable candidate for both cell separation and cell culture applications of magnetic levitation since it does not affect cell viability even at high concentrations (up to 100 mM) to levitate cells at high levitation heights [9, 16, 17, 22].
10. Other magnetic resonance imaging (MRI) contrast agents containing different ligand forms of gadolinium may also be used to increase magnetic susceptibility of the solution and provide the levitation of the cells in a magnetic field gradient. It should be noted that nonionic structure-containing agents, such as gadobutrol (Gadavist®, Bayer) and gadodiamide (Omniscan™, GE Healthcare), are more favorable for providing higher levitation heights than ionic structure-containing ones: gadopentetate dimeglumine (Magnevist®, Bayer), gadoterate meglumine (Dotarem®, Guerbet), and gadobenate dimeglumine (Multihance®, Bracco). Besides, for a better cell viability, macrocyclic ligand-containing agents (gadobutrol and gadoterate meglumine) are more suitable than linear ligand-containing agents (gadopentetate dimeglumine, gadodiamide and gadobenate dimeglumine) [16].
11. The other common paramagnetic salt, $MnCl_2$, may be used for manipulation of diamagnetic objects (e.g., cells) and density-based measurements [25, 26]. However, the long-term effect of the salt concentration required to levitate the cells on the target cells should be tested due to concerns of cell viability in cell culture applications [27].
12. The low concentrations of Gd^{3+} (~20–50 mM) are suitable for separation of cells with different densities [1, 9], while the high concentrations (~50–100 mM) are more suitable to prevent the descension of formed 3D constructs for cell culture applications [16, 17, 22].
13. For the single cell level analysis of the stem cells of bone marrow origin, a period of 10–15 min was found to be sufficient [1, 16]. Since this time may vary depending on the type of cell and the density of the solution, it would be useful to

determine the length of this period during the optimization process before the experiment.

14. In some cases, protective z-stage limit of the microscope prevents appropriate focusing to the center of capillary. Carefully manipulate that limit regarding the dimensional specifications of the hardware.
15. As the visualization is obtained via mirrors, a strong brightness is required to obtain a clear image.
16. The electrical or mechanical settings of microscope during imaging should be recorded for consistency of repetitive analyzes.
17. It is important that each cell or 3D living structure is seen separately and reflects the correct size of the particle (cell or 3D structure). If multiple particles are observed as a single particle when the settings that reflect correct size of the particles are completed, "watershed" tool can be used to divide adjacent particles into zones reflecting single particle size.
18. If a proper region of levitated cells is not selected, analysis will include debris and dead cells that are positioned at the bottom surface of the capillary. Based on the refraction at this surface, it is often challenging to perform an analysis. Therefore, if the intention is to measure the density of dead cells, it is advisable to manipulate magnetic susceptibility of the medium so that the dead cells are levitated as well.
19. The size and circularity limits in the "analyze particle" window of ImageJ Fiji software will be useful to exclude undesirable areas from the analysis. Although this setting needs to vary according to the size of the cells analyzed and size and clearness of the image, it is convenient to adjust size limits between 1 and 300 and circularity limits between 0.01 and 1 for stem cells of bone marrow origin displayed under 4× magnification in a 1600 × 1200-pixel image where 1 pixel is equal to 1.724 μm.
20. If the results are in pixels, perform the pixel-μm conversion.
21. At least three different density beads of known density should be selected for calibration within the expected density range of cells that will be measured (e.g., 1.01–1.10 g/mL).

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