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Original Research Article

Effect of enzymatic and mechanical methods of dissociation on neural progenitor cells derived from induced pluripotent stem cells

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ABSTRACT

Purpose: To determine the most effective method of dissociating neural stem and progenitor cells into a single-cell suspension.
Materials/methods: Induced pluripotent stem cells were differentiated toward the neural fate for 4 weeks before clusters were subjected to enzymatic (Accutase, trypsin, TrypLE, dispase, or DNase I) or mechanical (trituration with pipettes of varying size) or combined dissociation. Images of cells were analyzed for cluster size using ImageJ.
Results: Cells treated with the enzymes Accutase, TrypLE, or trypsin/EDTA, these enzymes followed by trituration, or a combination one of these enzymes followed by incubation with another enzyme, including DNase I, were more likely to be dissociated into a single-cell suspension.
Conclusions: Cells treated with enzymes or combinations of methods were more likely to be dissociated into a single-cell suspension.

1. Introduction

Neural stem and progenitor cells are in active use for a myriad of research applications, including the study of development, disease pathophysiology, drug and toxin screening, and grafting in animal models of neurological disorders. An obstacle to some of these applications is the fact that they often grow in large clusters that are difficult to dissociate without substantial cell loss, likely from loss of cell-cell contact or disruption of adherent cell processes in this cell population [1,2]. To avoid this cell loss, multiple practices have been developed to work around having to dissociate these clusters [3–5]. Complete dissociation into a single-cell suspension, however, is necessary for accurate cell counts, which can affect the reproducibility of results, assays involving flow cytometry, and studies of the impact of cell–cell interactions on survival and maturation of cells both *in vitro* and *in vivo* [6–8].

Current methods of dissociation include mechanical and enzymatic treatments. Mechanical dissociation methods include the use of filters, chopping techniques, microfluidic devices, and various trituration strategies using a variety of pipettes [2–5]. Enzymatic dissociation methods include the application of proteolytic enzymes such as trypsin, TrypLE, dispase, and Accutase, with or without also manipulating ion concentrations [1,4,7–10]. We sought to determine which method optimally balanced neural cell cluster dissociation with cell survival by directly comparing a wide range of mechanical, enzymatic, and combination dissociation methods.

2. Material and methods

2.1. Cell culture

Human induced pluripotent stem cells (iPS-DF6-9-9T) were maintained in a Heracell 240 humidified incubator (Heraeus) at 5% CO_2 and 37 °C. Cells were expanded in the pluripotent state and differentiated to neural lineages as previously described [11–13]. Briefly, pluripotent cells were expanded in 6-well plates (Nunc) on a feeder layer of irradiated mouse embryonic fibroblasts (WiCell) in 3 mL per well of proliferation media plus fibroblast growth factor 2 (PM + FGF2). PM + FGF2 is composed of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12) plus 2.5 mM L-glutamine and 15 mM HEPES Buffer (Fisher), 20% Knockout Serum replacement (Gibco), 1% minimum essential medium Eagle: non-essential amino acids (MEM-NEAA; Invitrogen), 1% penicillin-streptomycin (Invitrogen), 0.5% Glutamax-1000

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(Invitrogen), and 0.1 mM beta-mercaptoethanol (Sigma) plus 4 ng/mL FGF2 (R&D Systems). Cells were passaged every 7 days with 1 unit/mL dispase (Gibco) at 37 °C for 5 min followed by scraping to lift cells. Cells were centrifuged in an Eppendorf Centrifuge 5702 (Eppendorf) for 1 min, at 1000 rpm, and re-suspended in 6 mL PM. At each passage, 1/6 of the cells from each plate were kept for continued proliferation while the remaining 5/6 of the cells were started on the neural differentiation protocol. Proliferating cells were fed after 2 days, and every day thereafter until passaging, with PM + FGF2.

Differentiating cells were suspended in 15 mL PM (without FGF2) in 25 mL flasks (Nunc) for 2 days, allowing any remaining feeder cells to attach to the flask. On Day 3, cells were moved to a new flask and fed with PM. On Day 4, proliferation medium was replaced with neural medium (NM). NM is comprised of DMEM/F-12 with 2.5 mM L-glutamine and 15 mM HEPES Buffer, 1% MEM-NEAA, 1% penicillin-streptomycin, 1% N2 supplement (Invitrogen), and 2 mg/mL heparin (Sigma). On Day 5, cells were fed with NM. On Day 6, cells were re-suspended in 6 mL NM plus 10% fetal bovine serum (FBS; Gibco) and attached 1 mL per well of a 6-well plate for 18 h. NM + FBS was then removed and cells were fed with NM on Days 8 and 11–13. On Day 14, cells were gently lifted by blowing with a P1000 pipette and the detached clusters were grown in suspension in 25 mL flasks in NM until Day 32 or 33 when they were dissociated.

2.2. Dissociation

Cells from each flask were collected in 15 mL tubes (Dot Scientific), centrifuged 1 min, 1000 rpm, and re-suspended in 1 mL Dulbecco's modified Eagle's medium (DMEM; Fisher). In order to begin with samples containing the same number of cells, we chose to count the cells from each flask by dissociating a small portion of the cell suspension using Accutase Cell Detachment Solution (Fisher) prior to counting. 100 μL of the cell suspension was transferred to a new 15 mL tube with 1 mL Accutase, and incubated 10 min in a 37 °C H_2O bath. Cells were centrifuged 1 min, 1000 rpm, re-suspended in 1 mL DMEM, and the numbers of live and dead cells were counted using trypan blue solution (Sigma) and a hemocytometer (Fisher). Cells from flasks containing fewer than 500,000 cells were not included in the study.

The remaining cells were then re-suspended in DMEM at the volume necessary to get a concentration of 500,000 cells/mL and then divided into 1 mL aliquots for dissociation. All triturations for re-suspension in DMEM and/or enzyme were performed 3 times with a 5 mL Fisherbrand Sterile Polystyrene Disposable Serological Pipette (Fisher) unless otherwise indicated.

2.3. Enzymatic dissociation

The enzymes tested were Accutase (Acc), Gibco's TrypLE Express (1×) without phenol red (Invitrogen), Gibco's Trypsin/EDTA solution (Invitrogen), dispase (Invitrogen), and Type II Deoxyribonuclease I (DNase I) from bovine pancreas (Sigma). All enzymes were used at their supplied working concentrations, one lot tested per enzyme, except dispase and DNase I. Dispase was dissolved in DMEM to 1 unit/mL activity. DNase I was prepared in DMEM for a final concentration of 200 units/mL activity [14]. Single lots of dispase and DNase I stock enzyme were tested, but working concentrations were prepared as needed.

Cells were centrifuged, re-suspended in 1 mL enzyme, and incubated at 37 °C in a H_2O bath for 10, 20, or 30 min. Half-way through the incubation period, tubes were shaken lightly to re-suspend cells. Cells were then centrifuged and re-suspended in 1 mL DMEM. 50 μL cell suspensions was reserved for counting as described above; the remainder of the cell suspension was fixed and stained as described below.

2.4. Mechanical dissociation

Following a modification of the protocol outline by StemCell Technologies [15,16], we tested the efficacy of mechanical dissociation using Fisherbrand Redi-Tips 101–1000 μL blue and 10–200 μL yellow (Fisher) with the P1000 Pipetman Neo (P1000) and P200 Pipetman (P200), respectively (Gilson Inc.). Although this protocol suggested triturating for a total of 20–30 times, we chose to halve that to hopefully increase cell survival. Cells were centrifuged and 200 μL DMEM was added. Cells were then triturated 2 or 3 times at 200 μL by a single researcher maintaining a consistent speed of approximately 3 times in 2 s. Large clusters were allowed to settle before 180 μL were transferred to a new tube. Another 200 μL DMEM was added and cells were again triturated, settled, and transferred. The process was repeated for a total of 10 or 15 triturations and a final volume of 1 mL. 50 μL cell suspensions was reserved for counting as described above; the remainder of the cell suspension was fixed and stained as described below.

Additionally, we tested the common practice of breaking clusters with fire-polished 9-inch Pasteur pipettes (Fisher) [17]. Briefly, in the biosafety cabinet, the tip of the pipette was flamed with a Bunsen burner until the inner lumen was narrowed. The neck of the pipette was then heated until it bent to approximately 45° and the pipette was cooled to room temperature. Pipettes were rinsed with DMEM prior to dissociating the cells to prevent cells from sticking to the inner surface. The entire 1 mL cell suspension was triturated 3 or 5 times with the pipette following the protocol outlined by Hu and Zhang [17]. We also followed the above mechanical dissociation protocol for a total of 3, 5, 10 or 15 triturations with the fire-polished pipettes (FPP) to determine if this would yield a more dissociated sample than the P200 or P1000 pipettes. 50 μL cell suspensions was reserved for counting as described above; the remainder of the cell suspension was fixed and stained as described below.

2.5. Combination dissociation protocols

For enzyme combinations, cells were centrifuged, re-suspended in 1 mL enzyme, and incubated at 37 °C in a H_2O bath for 10 or 20 min. Cells were then centrifuged and re-suspended in 1 mL of the next enzyme and incubated at 37 °C in a H_2O bath for 10 min. Half-way through both incubation periods, tubes were shaken lightly to re-suspend cells. Cells were then centrifuged and re-suspended in 1 mL DMEM. 50 μL cell suspensions was reserved for counting as described above; the remainder of the cell suspension was fixed and stained as described below.

For combinations of enzymatic and mechanical dissociation methods, cells were first treated with the indicated enzyme as described above for 10 min. Cells were then centrifuged and re-suspended in 200 μL DMEM and then treated to 10 or 15 triturations with the P200 or P1000 pipette as described above with a final volume of 1 mL cell suspension. 50 μL cell suspensions was reserved for counting as described above; the remainder of the cell suspension was fixed and stained as described below.

2.6. Fixation and staining

Prior to fixation and staining, 50 μL of the cell suspension was removed and cells were counted as described above. From this point on, all triturations to re-suspend cells were performed 3 times with a P1000 pipette unless otherwise indicated. The remainder of the cell suspension was centrifuged for 10 s,