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

Lindsey D. Jager, PhD^a, Claire-Marie A. Canda^a, Crystal A. Hall^a, Cassandra L. Heilingoetter^a, Joann Huynh^a, Susanna S. Kwok^a, Jin H. Kwon^a, Jacob R. Richie^a, and Matthew B. Jensen, MD, MS^{a,*}

^aDepartment of Neurology, University of Wisconsin, Madison, WI, USA

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.

Keywords

Stem cells; neural progenitor cells; dissociation; 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,

*Corresponding author at: Department of Neurology, University of Wisconsin-Madison, 1685 Highland Ave #7273, Madison, WI 53705-2281. Tel.: +1-608-265-4351; Fax: +1-608-263-0412. jensen@neurology.wisc.edu..

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% CO₂ 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 15mM 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 (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 minutes followed by scraping to lift cells. Cells were centrifuged in an Eppendorf Centrifuge 5702 (Eppendorf) for 1 minute, 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 hours. 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 minute, 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 uL of the cell suspension was transferred to a new 15 mL tube with 1 mL Accutase, and incubated 10 minutes in a 37°C H₂O bath. Cells were centrifuged 1 minute, 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 (1x) 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. Dispace 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 H₂O bath for 10, 20, or 30 minutes. 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 uL 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 uL blue and 10-200 uL 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 uL DMEM was added. Cells were then triturated 2 or 3 times at 200 uL by a single researcher maintaining a consistent speed of approximately 3 times in 2 seconds. Large clusters were allowed to settle before 180 uL were transferred to a new tube. Another 200 uL 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 uL

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 uL 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 H₂O bath for 10 or 20 minutes. Cells were then centrifuged and re-suspended in 1 mL of the next enzyme and incubated at 37°C in a H₂O bath for 10 minutes. 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 uL 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 minutes. Cells were then centrifuged and re-suspended in 200 uL 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 uL 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 uL 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 seconds, 6000 rpm in a Fisherbrand standard mini-centrifuge (Fisher). The supernatant was removed and cells were re-suspended in 500 uL PBS and triturated to separate cells from the pellet. 500 uL of 8% paraformaldehyde (Sigma) were then added (final concentration 4%) and the cells were triturated again. The cell suspension was incubated at room temperature for 15 minutes. The cells were centrifuged and the supernatant was removed; cells were re-suspended in 1 mL PBS, triturating only 1 time to preserve the cell membranes, and stored at 4°C overnight.

The nuclei were stained using Hoechst 33342, trihydrochloride, trihydrate (Invitrogen). Briefly, cells were centrifuged and re-suspended in 1 mL PBS plus Hoechst (1:1000), triturating 1 time, covered and incubated at room temperature for 15 minutes. Cells were

centrifuged and as much as possible of the supernatant was removed. 2.5 uL PBS was added using a P20 Pipetman Neo (Gilson) and cells were triturated 3 times. 2.5 uL Fluoromount G (Southern Biotech) was added and the cells were triturated an additional 3 times taking care not to introduce bubbles. To collect the cells for mounting on the slides, the P20 was set to 5 uL and the plunger was depressed to the second stop; cells were drawn up into the pipette by fully releasing the plunger. To avoid bubbles, a 5 uL droplet of cell suspension was placed on a Fisherbrand ColorFrost Plus Blue slides (Fisher) by depressing the plunger to the first stop (but not the second). Fisherbrand 12 mm round glass coverslips (Fisher) were then gently placed on the droplet. Slides were protected from light and allowed to dry overnight before being stored at 4°C until imaging.

2.7. Imaging

Images of the whole coverslip were taken using a Nikon A1R-Si laser scanning confocal microscope (Nikon) with NIS Elements Advanced image processing software (Nikon). Individual images taken at 10X magnification using the 405 laser, gain set to 100, were stitched together with an overlap of 10%. The pixel:micron ratio is 0.1997.

2.8. Image analysis

Analysis was performed using ImageJ [18]. The blue channel image was converted to a mask using the Renyi Entropy thresholding equation. The mask was then processed to fill in the centers of large clusters using the Dilate and Fill Holes functions. Finally, the area around the coverslip was cleared to avoid analyzing any debris or portions of other coverslips that may have been included in the image. The individual particles were then counted and their areas were measured using the Analyze Particles function. Circularity was set at 0-1.00. The average size of a single cell is approximately 20 pixel² (501.7 um²) after image processing. We analyzed multiple coverslips to determine the optimum size range necessary for counting the maximum number of single cells while eliminating false positives. We considered any particles with an area of 0-19 pixel² (0-476.6 um²) to be debris and they were excluded from our measure. The total number of particles between 20-INFINITY pixel² was counted and the average particle size was calculated.

2.9. Statistical analysis

A minimum of three coverslips were analyzed per group. Groups were compared using repeated measures one-way ANOVA using GraphPad Prism version 6.03 for Windows (GraphPad Software, La Jolla California USA). Unpaired two-tailed Student's t-tests were then performed to compare the control group to those groups whose means fell within the range of countable single cells and small groups of cells, defined by the authors as between 20-150 pixel² (500-3750 um²).

3. Results

The different dissociation methods produced cell suspensions including single cells, groups of double and triple cells, and clusters ranging from small collections of 10-20 cells to large clumps of cells visible to the naked eye ($p < 0.0001$) (Figure 1). Untreated cells had an average particle size of over 17000 um². We defined dissociated samples as being composed

of single cells and small groupings of cells where the individual cells were easily distinguished, with a particle size of 500-3750 μm^2 . As can be seen in Figure 2, none of the mechanical methods for dissociation produced an average particle size within the target range. Four of the groups treated with enzymes followed by mechanical dissociation did. Accutase followed by 10 or 15 triturations with the P200 pipette produced samples with a mean particle size of 2937.2 and 2620.5 μm^2 , respectively. Samples treated with Trypsin/EDTA (TE) followed by 10 or 15 triturations with the P200 pipette averaged 1802.2 and 2720 μm^2 , respectively. Four of the single-enzyme treatment groups, including incubation with Accutase and TrypLE for 10 minutes and Accutase and Trypsin/EDTA for 30 minutes also produced dissociated samples. Ten of the groups using enzyme combinations also produced dissociated samples. The smallest mean particle size (1575.2 μm^2) was obtained using a combination of TrypLE followed by Trypsin/EDTA. When compared to the untreated control group, the average particle size of each of these dissociated groups was found to be significantly smaller ($p < 0.05$).

The total number of cells counted for each sample after dissociation varied widely for each group, despite the fact that each sample started with an estimated 500,000 cells (Figure 3). Cell counts for undissociated or minimally dissociated groups were as low as 2857 cells for the Control group. The group treated with Accutase for 10 minutes followed by TrypLE for 10 minutes had the highest average total cell count, at 836,000 cells, despite having an average particle size outside of our defined range of dissociation (4572.6 μm^2). For groups that met our criteria for dissociation, the total cell counts ranged from 227,500 cells in the TrypLE for 10 minutes group to 646,600 cells for the group treated with Trypsin/EDTA for 10 minutes followed by Accutase for 10 minutes. The groups with the lowest cell counts also had the lowest viabilities (Figure 4). Viability ranged from 10% for the group treated with 15 triturations by a fire-polished pipette (15,000 cells) to 48.6% for samples treated with dispase for 10 minutes (6000 cells). Of the methods that produced samples that met our criteria for dissociated samples, the lowest viability was seen in cells treated with Trypsin/EDTA for 30 minutes (60.8%). Triturating samples after enzyme treatment yielded samples with viabilities from 70 to 80%. The highest viability calculated was 91.3% from samples treated with TrypLE for 10 minutes followed by either DNase I or Trypsin/EDTA for 10 minutes. However, low cell counts and low viabilities are not necessarily due to cell death, as many samples had large, visible cell pellets after centrifugation (Figure 5) and/or cells trapped in large gelatinous masses (Figure 6), both of which likely yielded inaccurate cell counts.

4. Discussion

Trituration and enzymatic methods have the advantages of being easy to use, allow minimal chance of contamination, and require no specialized equipment, although the cost of enzymes may be prohibitive for laboratories engaging in the large-scale production of stem cells [7]. While not the first study to broach the subject of how to best create a single-cell suspension, ours is unique in that we compared multiple methods of enzymatic dissociation at multiple time points alone and in combination with mechanical methods that are commonly used.

We chose to use average cluster size as the primary outcome for our study because our goal was to determine how well each method dissociates the clusters into a single-cell suspension. We defined dissociated samples as being composed of single cells and small groupings of cells where the individual cells were discernible. Particles of 500-3750 μm^2 matched the criteria. Enzymes proved to be better at producing dissociated samples than mechanical methods. Treatment with Accutase, which contains proteolytic and collagenolytic enzymes, Trypsin/EDTA, a serine protease, and TrypLE, a recombinant serine protease, yields dissociated samples whether used alone or in combination with mechanical methods or another enzyme. Adhering strictly to our criteria, it would appear that treating cells with TrypLE for 10 minutes followed by Trypsin/EDTA for 10 minutes would be the best choice.

However, there are many other factors that need to be considered, such as the viability of the cell population post-dissociation. Prior to fixation and staining, we counted the number of live and dead cells and calculated viability. It was clear that the efficacy of the dissociation method strongly impacted the ability to count the cells and, thus, measure viability. As can be seen in Figure 3, although all samples started with approximately 500,000 cells, the mean cell number in the un-dissociated and minimally dissociated samples was around 5-10,000. For the minimally dissociated groups with larger clusters, this was not necessarily due cell death, as the cell pellets observed upon centrifugation were quite large even though the final cell counts were small, but because the cells remained in large clusters that were impossible to count (Figure 5). Viability thus ranged from 10% to over 90% depending on the method of dissociation (Figure 4).

An oddity that arose during our study was the propensity of cells treated with TrypLE and/or Trypsin EDTA to aggregate into large, viscous gelatinous-like masses with readily visible single cells and clusters (Figure 6). The longer the cells stayed in the dissociation solution, or if the cells were treated with both TrypLE and Trypsin/EDTA, the more viscous the sample became. These masses did not readily break apart upon trituration, but seemed to dissipate over the course of the fixation and staining process. Panchision, et al., describe how treatment with TrypLE leads to the presence of free DNA, and a technical communication from Stem Cell Technologies suggests that free DNA can lead to cell clumping [6, 14]. When followed by treatment with DNase I, these masses dissolved, yielding samples with both good dissociation and high viability [6, 14]. Therefore, we would not recommend treating cells with either TrypLE or Trypsin EDTA alone unless they are to be immediately fixed.

The strengths of our study are that we evaluated a number of widely available and commonly used dissociation methods, alone and in combination, and that we quickly fixed and analyzed our samples via cytological examination strictly for their average cluster size post-dissociation. Because we did not culture our cells after dissociation, we were able to avoid potentially losing cells and cell clusters during the seeding, feeding, and subsequent staining methods that are commonly used for studying stem cells, as well as avoid any proliferation that may occur in culture and increase cluster size.

However, our study does have weaknesses. For example, the number of triturations samples were subjected to during the fixation, staining, and mounting procedures may have further dissociated each sample. The small volumes required by the nature of our coverslipping method, only 5uL per sample before the coverslips began floating, allow for the possibility that some single cells and clusters were excluded. This could potentially be avoided by using flow cytometry to analyze cluster size; however, larger clusters can clog the instrument and the shear forces encountered during the process can lead to cell damage and death, which could then skew the results [9, 19]. Cells were aliquoted into separate samples of roughly 500,000 cells for testing following a pre-count of the number of cells in the flask using Accutase for 10 minutes. It is possible that some samples may have received larger or smaller clusters than others, skewing the results. While using ImageJ's automated particle counter allowed us to both count and measure the size of clusters on an entire coverslip, this method relies on the software selectively including and excluding pixels at a set level of intensity, therefore, high background staining and brightly-fluorescing debris may create particles that are counted and measured as cells. Additionally, we studied only a single cell line. Investigators using different cell lines, cells from fetal or embryonic tissue, and cells differentiated using other protocols may find other combinations of dissociation methods to be more effective. We did not include mechanical methods of dissociation involving filters, choppers, and/or microfluidic devices that may not be readily available to most laboratories [4, 5, 8]. Finally, we did not evaluate the dissociation methods for their effects on the long-term survivability and maturation of our cells, as this was beyond the scope of our study. Enzymatic dissociation is known to have many effects on survivability and maturation, including such parameters as ability to re-attach post-dissociation, increased apoptosis, increased need for small molecules such as Rho-associated protein kinase (ROCK) inhibitor to prevent apoptosis-induced cell loss, loss of cell-surface antigens, and effects on karyotype stability [1, 2, 6, 7, 20]. Therefore, we caution investigators to further examine the long-term effects of each dissociation method before selecting the one that best suits their needs.

In conclusion, our study suggests that enzymes dissociate stem cell clusters more thoroughly than mechanical methods, and that combining methods yields greater dissociation.

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Figure 1.

Representative images of dissociated cells. A. Untreated (average particle size for this image: $18215.3 \mu\text{m}^2$) B. Mechanical dissociation 10 times with P1000 (average particle size for this image: $18172.6 \mu\text{m}^2$) C. Enzymatic dissociation 20 minutes with TrypLE (average particle size for this image: $5177.1 \mu\text{m}^2$) D. Combined enzymatic and mechanical dissociation incubating with TrypLE for 10 minutes followed by 10 triturations with P1000 (average particle size for this image: $3935.5 \mu\text{m}^2$) E. Combined enzymatic dissociation incubating with TrypLE for 10 minutes followed by DNase I for 10 minutes (average particle size for this image: $1084.5 \mu\text{m}^2$). Scale bar: 1mm.

Figure 2.

Average particle size (in μm^2) following dissociation. Coverslips were analyzed with ImageJ's Analyze Particles function to determine the size of each single cell, group of cells, or cell clusters. Dissociated samples are defined as being composed of single cells and small groupings of cells where the individual cells were easily distinguished, with a particle size of $500\text{--}3750 \mu\text{m}^2$. * $p < 0.05$ when compared to untreated cells (control). Abbreviations: Acc, Accutase; FPP, fire-polished Pasteur pipette; P200, P200 Pipetman; P1000, P1000 Pipetman; TE, Trypsin/EDTA. Data show mean $\pm$ SD.

Figure 3. Total live cell count by hemocytometer after dissociation. Abbreviations: Acc, Accutase; FPP, fire-polished Pasteur pipette; P200, P200 Pipetman; P1000, P1000 Pipetman; TE, Trypsin/EDTA. Data show mean $\pm$ SD.

Figure 4. Cell viability. Both live and dead cells were counted after staining with trypan blue. Viability was calculated as the number of live cells divided by the total of live and dead cells. Abbreviations: Acc, Accutase; FPP, fire-polished Pasteur pipette; P200, P200 Pipetman; P1000, P1000 Pipetman; TE, Trypsin/EDTA. Data show mean $\pm$ SD.

Figure 5.

Presence of a large cell pellet but the sample yielded a low cell count. This suggests that the low cell counts and concurrent low viabilities calculated for many of the samples may not be due to cell death but due to poor dissociation yielding inaccurate cell counts. Samples were scored a 1 if there was a low cell count (<50,000 cells) despite the presence of a large cell pellet upon centrifugation and scored a 0 for all other conditions. Abbreviations: Acc, Accutase; FPP, fire-polished Pasteur pipette; P200, P200 Pipetman; P1000, P1000 Pipetman; TE, Trypsin/EDTA. Data show mean ± SD.

Figure 6.

Presence of gelatinous masses. Some dissociation methods triggered cells to aggregate into large, viscous gelatinous-like masses with readily visible single cells and clusters. These masses did not readily break apart upon trituration, thus may have contributed to low cell counts and subsequently low viabilities, but seemed to dissipate over the course of the fixation and staining process. Note the lack of gelatinous masses after combination treatments including DNase I. Samples were scored a 1 if a gelatinous mass was present following dissociation and scored a 0 if no gelatinous mass was present. Abbreviations: Acc, Accutase; FPP, fire-polished Pasteur pipette; P200, P200 Pipetman; P1000, P1000 Pipetman; TE, Trypsin/EDTA. Data show mean $\pm$ SD.

Table 1

Methods of Dissociation

Enzymatic	Mechanical	Combinations
Acc 10 min	P200 10x	Acc 10 min + P200 10x
TrypLE 10 min	P200 15x	Acc 10 min + P200 15x
TE 10min	P1000 10x	TrypLE 10 min + P200 10x
Dispase 10 min	P1000 15x	TrypLE 10 min + P200 15x
DNase I 10 min	FPP 3x	TE 10 min + P200 10x
Acc 20 min	FPP 5x	TE 10min + P200 15x
TrypLE 20 min	FPP 10x	Dispase 10 min + P200 10x
TE 20min	FPP 15x	Dispase 10 min + P200 15x
Dispase 20 min		DNase I 10 min + P200 10x
DNase I 20 min		DNase I 10 min + P200 15x
Acc 30 min		Acc 10 min + P1000 10x
TrypLE 30 min		Acc 10 min + P1000 15x
TE 30min		TrypLE 10 min + P1000 10x
Dispase 30 min		TrypLE 10 min + P1000 15x
DNase I 30 min		TE 10 min + P1000 10x
		TE 10min + P1000 15x
		Dispase 10 min + P1000 10x
		Dispase 10 min + P1000 15x
		DNase I 10 min + P1000 10x
		DNase I 10 min + P1000 15x
		Acc 10 min + TrypLE 10 min
		Acc 10 min + TE 10 min
		Acc 10 min + Dispase 10 min
		TrypLE 10 min + Acc 10 min
		TrypLE 10 min + TE 10 min
		TrypLE 10 min + Dispase 10 min
		TE 10 min + Acc 10 min
		TE 10 min + TrypLE 10 min
		TE 10 min + Dispase 10 min
		Dispase 10 min + Acc 10 min
		Dispase 10 min + TrypLE 10 min
		Dispase 10 min + TE 10 min
		Acc 10 min + DNase I 10 min
		TrypLE 10 min + DNase I 10 min
		TE 10 min + DNase I 10 min
		Dispase 10 min + DNase I 10 min
		Acc 20 min + DNase I 10 min

Enzymatic	Mechanical	Combinations
		TrypLE 20 min + DNase I 10 min
		TE 20 min + DNase I 10 min
		Dispase 20 min + DNase I 10 min

Abbreviations: Acc, Accutase; FPP, fire-polished Pasteur pipette; P200, P200 Pipetman; P1000, P1000 Pipetman; TE, Trypsin/EDTA.