Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

Title: Mechanical dissociation of human embryonic stem cell colonies by manual scraping after collagenase treatment is much more detrimental to cellular viability compared to trypsinization with gentle pipetting

Key Words : collagenase, embryonic, human, scraping, stem cells, trypsin, viability

Manuscript classification : Technical note / Short communication

Authors : 1) **Boon Chin Heng**
Stem Cell Laboratory
Faculty of Dentistry, National University of Singapore
5 Lower Kent Ridge Road, 119074 Singapore

2) **Hua Liu**
Stem Cell Laboratory
Faculty of Dentistry, National University of Singapore
5 Lower Kent Ridge Road, 119074 Singapore

3) **Zigang Ge**
Stem Cell Laboratory
Faculty of Dentistry, National University of Singapore
5 Lower Kent Ridge Road, 119074 Singapore

4) **Tong Cao**
Stem Cell Laboratory
Faculty of Dentistry, National University of Singapore
5 Lower Kent Ridge Road, 119074 Singapore

Correspondence and reprint requests : **Dr. Tong Cao**
Stem Cell Laboratory
Faculty of Dentistry
National University of Singapore
5 Lower Kent Ridge Road,
Singapore 119074
e-mail : dencaot@nus.edu.sg
Tel : +65-6516-4630
Fax : +65-6774-5701

Requirement for color printing : none

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

Abstract

Because human embryonic stem cells (hESC) are 'social cells' that require cooperative interactions and intimate physical contact with each other, it is absolutely essential to dissociate hESC colonies into cellular clumps rather than into a single cell suspension during serial passage. This study compared two commonly-used protocols for dissociating hESC colonies. The first protocol involved mild enzymatic treatment with collagenase type IV (1 mg/ml) for about 5 to 10 min, prior to mechanical dissociation into cellular clumps through manual scraping with a plastic pipette tip. The second protocol involved a short duration of exposure (2 to 3 min) to low concentrations of trypsin (0.05%), followed by gentle pipetting. The MTT assay was used compare the recovery of viable cells after dissociating hESC colonies with these two protocols, before and after conventional freeze-thawing with 10% (v/v) DMSO. Besides undifferentiated hESC, the randomly differentiated fibroblastic progenies of hESC at various passages (P0 to P4) together with an immortalized cell line (CRL-1486) were also utilized to compare the two protocols. The results demonstrated that the second protocol (trypsinization with gentle pipetting) is much less detrimental to cellular viability compared to the first protocol (collagenase treatment with scratching). This in turn translated to higher freeze-thaw survival rates. It is hypothesized that scratching after collagenase treatment (first protocol) somehow induces physical damage to the cells thereby leading to a lower recovery of viable cells, both before and after freeze-thawing.

Key Words : collagenase, embryonic, human, scraping, stem cells, trypsin, viability

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

Introduction

Human embryonic stem cells (hESC) are pluripotent cells derived from the inner cell mass of blastocyst stage embryos [1-3]. As such, they hold much promise not only in the newly emerging field of regenerative medicine [4], but also as a tool in basic scientific research, and for in vitro pharmacological and toxicology screening [5, 6]. An essential prerequisite for these widespread applications are their efficient propagation in vitro.

Currently, there is much variation in the protocols developed for routine serial passage and culture of the various hESC lines that have been isolated [1-3]. Besides several notable differences in the culture milieu formulation [7], the various protocols also differ in the method of dissociating hESC colonies into cellular clumps for seeding onto fresh murine embryonic fibroblast (MEF) layers [1-3]. Unlike murine embryonic stem cells, hESC are 'social cells' that require cooperative interactions and initimate physical contact with each other to maintain their viability and self-renewal capacity within in vitro culture [7]. Hence, it is absolutely essential to dissociate hESC colonies into cellular clumps rather than into a single cell suspension during routine serial passage. A cell-clump size of about 100 to 200 cells was reported to be optimal for hESC colony growth and propagation [1, 2].

The first reported technique for dissociating hESC colonies during serial passage was reported by Thomson et al. [1], and involved mild enzymatic treatment with collagenase type IV (1 mg/ml) for about 5 to 10 min, prior to mechanical dissociation into cellular clumps through manual scraping with a plastic pipette tip. This was followed by the technique of mechanically dissecting individual hESC colonies under a steromicroscope, which also involved mild enzymatic treatment with dispase [2]. Subsequently, Cowan et al. [3] developed a technique of enzymatically dissociating hESC colonies into appropriate-sized cellular clumps without the need for mechanical dissociation. This was achieved by short duration of exposure (2 to 3 min) to low concentrations of trypsin (0.05%), followed by gentle pipetting (figure 1).

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

Currently, either one of these three techniques or a slight variation of them are most commonly employed for the serial passage of the various hESC lines that have isolated worldwide. Hence, the pertinent question that arises is which one of these three techniques is the most efficient for maximal recovery of hESC during serial passage, since it is likely that enzymatic treatment together with physical stress encountered during mechanical dissociation can be detrimental to cellular viability.

Of the three techniques discussed, the mechanical dissection of individual hESC colonies under steromicroscopy developed by Reubinoff et al. [2], appears to be too tedious and labor-intensive for large-scale culture of hESC, and will therefore not be further examined in this study. Hence, this would leave us to compare the other two remaining techniques: collagenase treatment followed by mechanical dissociation [1], and trypsinization with gentle pipetting [3]. Both these two methods of dissociating hESC colonies have been demonstrated to be effective in preserving stem cell pluripotency during prolonged in vitro culture. In the original study of Thomson et al. [1], it was reported that the undifferentiated pluripotent state of four different hESC lines (H1, H9, H13 & H14) was maintained after four to five months (20 to 30 passages) of continuous culture with the technique of collagenase digestion and manual scraping during serial passage. Similarly, Cowan et al. [3] reported that the undifferentiated pluripotential state of five hESC lines (HUES-1 to -4 and HUES-6) was maintained after 50 passages, with the technique of mild trypsinization and gentle pipeting during serial passage. Hence, this study will not attempt to further investigate the effects of passaging technique on the long-term pluripotency of hESC.

By utilizing the MTT (Tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [8], this study will attempt to measure the percentage recovery of viable cells after dissociating hESC colonies with these two techniques. Originally, the MTT assay was developed for adherent cells. Hence, this study will attempt to modify the assay for a suspension of cellular clumps. The other alternative may be to utilize a non-permeant fluorescent dye like propidium iodide which binds only to DNA of dead cells, and then do a quantitative analysis with flow cytometry. Nevertheless, this is inapplicable to this study because flow cytometry analysis would require a single-cell suspension, which

would make it impossible for us to compare dissociation of hESC colonies into cellular clumps by the two different passage techniques developed by Thomson et al. [1] and Cowan et al. [3].

Besides undifferentiated hESC colonies, this study will also compare the two techniques with respect to monolayers of randomly differentiated hESC fibroblastic progenies at different passages (up to passage 4), together with an immortalized cell line (CRL-1486) as a comparative control. Additionally, the two techniques will also be compared with respect to the survival rate upon conventional freeze-thawing of the undifferentiated hESC and differentiated hESC fibroblastic progenies.

Materials and Methods

Cell lines, Media, Reagents & Chemicals

The hESC were obtained from the Wicell Research Institute Inc. (Madison, WI, USA), and were of the H1 line listed on the National Institute of Health (NIH) registry, which had received Federal approval for United States government-supported research funding [9]. CRL-1486 cell line (derived from human embryonic palatal mesenchyme) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Unless otherwise stated, all liquid media, serum or serum replacement was purchased from Gibco BRL Inc. (Gaithersburg, MD, USA), all labware consumables were purchased from Becton-Dickinson Inc. (Franklin Lakes, NJ, USA), while all other reagents and chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Culture and propagation of hESC in the undifferentiated state

Undifferentiated hESC were maintained on a feeder layer of mitomycin C-inactivated murine embryonic fibroblast feeder (MEF) cells [1, 2]. These were harvested from CF1 inbred

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

mouse strain purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). The culture medium was DMEM/F12 supplemented with 20% (vol/vol) Knockout (KO) serum replacement, 1mM L-glutamine, 1% nonessential amino acid, 100mM β-mercaptoethanol and 4ng/ml bFGF. All cell cultures were carried out on 6-well culture dishes (Nunc Inc., Roskilde, Denmark) within a humidified 5% CO_2 incubator set at 37^{o}C. The culture media was changed daily with routine passage of hESC on a fresh MEF layer being carried out once a week. Unless otherwise stated, dissociation of hESC colonies during serial passage was achieved by treatment with 1 mg/ml collagenase type IV for between 5 to 10 min, following by mechanical scraping with a plastic pipette tip, according to the protocol recommended by Wicell Reseach institute Inc. [1].

Random free differentiation of hESC into fibroblastic progenies

hESC clumps (100-400 cells) fresh from serial passage were seed onto porcine gelatin-coated 6-well culture dishes (surface area of 1 well of a 6–well dish ≈ 9.6 cm^2). The culture milieu composed of Dubelcco's minimum essential medium (DMEM) supplemented with 1 mM L-glutamine and 10% (v/v) fetal calf serum (FCS). Approximately 2 to 4 million cells (about 2 confluent wells) were seeded onto a single 6-well dish. 7 days after initial seeding of the hESC clumps, a confluent monolayer was formed (labeled as P0), which was either utilized for experiments or trypsinized and passaged at a split-ratio of 1:4 into 6-well dishes (non gelatin-coated). Subsequent serial passages were carried out once every 7 days at the same split-ratio, up to passage 4 (P4, 35 days of random free differentiation).

Dissociation of hESC colonies by two different protocols

The undifferentiated hESC, together with their differentiated fibroblastic progenies at different passages (P0 to P4, 7 to 35 days of random free differentiation) were dissociated into cellular clumps by two different protocols. The first protocol [1] involved mild enzymatic treatment with collagenase type IV (1 mg/ml) for about 5 to 10 min, prior to mechanical dissociation into cellular clumps through manual scraping with a plastic pipette tip. The second protocol [3] involved 2 to 3 min of exposure to a 0.05% trypsin solution,

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

followed by gentle pipetting (figure 1). The CRL-1486 cell line was utilized as a comparative control, and was subjected to the same treatment. The cellular suspension obtained by these two protocols was either subjected immediately to the MTT assay, or was frozen-thawed prior to the MTT assay. The cell count for each experimental group was determined by trypsinizing a known volume aliquot into a single-cell suspension, followed by tryphan blue staining and manual counting with a hemocytometer under bright-field microscopy. Altogether, there were 6 replicates for each experimental group.

Conventional freeze-thawing with 10% (v/v) Dimethyl Sulfoxide (DMSO)

After subjecting undifferentiated hESC or their differentiated fibroblastic progenies (P0 to P4) to the two different protocols, the cells were pelleted down by centrifugation (300g for 10 min) and then resuspended in 2ml of freezing solution constituting 80% (v/v) DMEM supplemented with 10% (v/v) FCS and 10% (v/v) Dimethyl Sulfoxide (DMSO), within 15 ml falcon tubes [10]. This was followed by slow freezing within a Styrofoam box placed in a -80^{o}C refrigerator. For each experimental group, the number of cells that were subjected to freeze-thawing corresponded to the exact number being subjected directly to the MTT assay. After at least 1 week of storage at -80^{o}C, thawing was achieved by placing the tubes within a 37^{o}C water bath, followed by a single wash in DMEM (300g centrifugation for 10 min) prior to the MTT assay.

Modified MTT assay for cellular suspension

The MTT (Tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [8] was performed to quantify and compare the recovery rate of viable cells after treatment with the two different protocols, before and after freeze-thawing (Figure 2 & 3). Because the MTT assay was originally developed for adherent cells, this would need some modification and adaptation for a suspension of cellular clumps. This involved placing the cellular suspension within a 15 ml falcon tube followed by centrifugation at 300g for 10 min. The supernatant was then removed and the cell pellet was re-suspended and dispersed in 2ml of MTT solution (1 mg/ml in 90% (v/v) DMEM with 10% (v/v) FCS). The cap of the 15 ml

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

tubes were then tightened and sealed with parafilm, prior to placing them horizontally (sideways) within a 37^{o}C incubator, where they were incubated for 4h. Otherwise, placing the tubes vertically would lead to the cells sinking downwards, and there will be non-uniform exposure to MTT. After that, the cells were washed first in formol-calcium [0.4% (v/v) formaldehyde and 1% (w/v) anhydrous $CaCl_2$] followed by phosphate buffered saline (PBS) through centrifugation at 800g for 10 min, and then left to air-dry as a pellet at the bottom of the tube. The MTT-formazan products were extracted in the dark at room temperature with 2 ml of DMSO added to each 15 ml tube. 200 μl of the supernatant was later transferred into a 96-well flat-bottomed cell culture plates, and the absorbance readings were measured spectrophotometrically at 570 nm using a Sunrise modular microplate reader (Tecan, Maennedorf, Switzerland). From the absorbance values, the recovery rate of viable cells with the two different protocols, before and after freeze-thawing can then be compared.

Results

Freeze-thaw survival rate with protocol 1: collagenase treatment with mechanical dissociation through scratching with plastic pipette tip

For undifferentiated hESC, the freeze-thaw survival rate assayed by MTT was 28.9% with protocol 1. Upon random free differentiation, there was observed to be a gradual increase in the freeze-thaw survival rate from 35.9% at P0 to 75.0% at P3, which then dipped to 59.5% at P4 (table 1). By contrast, the freeze-thaw survival rate of the CRL-1486 cell line with protocol 1 was much higher at 91.0%.

Freeze-thaw survival rate with protocol 2: trypsinization with gentle pipetting

For undifferentiated hESC, the freeze-thaw survival rate assayed by MTT was 70.5% with protocol 2. Upon random free differentiation, the freeze-thaw survival rate was consistently

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

around 90% from P0 to P4 (table 1), which was somewhat similar to the survival rate of 92.6% obtained for the CRL-1486 cell line with protocol 2.

Percentage recovery of viable cells in protocol 1 as fraction of protocol 2 (without freeze-thawing)

In all experimental groups observed, the recovery of viable cells in protocol 1 is consistently lower than that of protocol 2. For undifferentiated hESC, the percentage recovery of viable cells in protocol 1 as fraction of protocol 2 (without freeze-thaw) is 58.0%, which is rather similar to the value of 53.2% obtained for the CRL-1486 cell line. By contrast, the corresponding values for the differentiated hESC fibroblastic progenies (P0 to P4) were much lower, ranging from 25% to 30% (table 1).

Discussion

The results of this study demonstrated conclusively that the method of dissociating hESC colonies during serial passage has a profound effect on cellular viability, as well as freeze-thaw survival rate. This in turn could have useful implications for the development of new protocols for the scale-up of hESC culture, which would certainly be required to provide bulk quantities of cells for therapeutic and non-therapeutic applications.

It appears that trypsinization with gentle pipetting [3] is much less detrimental to hESC and their differentiated fibroblastic progenies, as compared to collagenase treatment followed by mechanical dissociation through scratching with a plastic pipette tip [1]. This was confirmed by consistently higher absorbance readings in the MTT assay, which would indicate a higher recovery yield of viable cells (table 1). Interestingly, the freeze-thaw survival rates after trypsinization with gentle pipetting, are also consistently higher compared to collagenase with scratching (table 1).

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

A likely possibility is that scratching with a plastic pipette tip somehow compromises cellular viability by inducing physical damage to the cell. Monolayers of differentiated hESC fibroblastic progenies (P0 to P4) appears to be more vulnerable to such damage compared to multi-layered undifferentiated hESC colonies, as seen by the greater disparity in the yield of viable cells between the two protocols, as indicated by the MTT absorbance values (table 1). It is hypothesized that because cells are tightly aggregated and compacted together within multi-layered hESC colonies, these easily come off as a relatively big 'clump' or 'sheet' upon scratching with a pipette tip. Hence, they are somehow 'less harmed' by physical contact of the pipette tip with the culture dish surface. By contrast, cells within a single monolayer are not tightly aggregated together, and hence do not easily come off as a big 'clump' or 'sheet' upon scratching. Instead, they would come off as aggregates of a few cells (figure 3A). From our practical experience, more extensive scratching is required to completely disperse a monolayer into a cellular suspension, as compared to multi-layered compact colonies; which would mean that a higher degree of physical damage is inflicted to cells within a monolayer by scratching. This in turn translates to consistently lower freeze-thaw survival rates for the differentiated hESC fibroblastic progenies (P0 to P4). The observed higher survivability of CRL-1486 cells upon scratching of the monolayer before and after freeze-thawing may be due to their different physiology as an immortalized somatic cell line.

Hence in conclusion, our study clearly demonstrated that the technique of dissociating hESC colonies by trypsinization and gentle pipetting, first reported by Cowan et al. [3], is less detrimental to cellular viability compared to the technique of collagenase treatment and scratching [1]. To our knowledge, this is also the first study to apply the MTT assay to a cellular suspension, as opposed to a layer of adherent cells.

References

1. Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. (1998) Science. **282**, 1145-1147

2. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. and Bongso, A. (2000) Nat. Biotechnol. **18**, 399-404

3. Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D. and Melton, D.A. (2004) N. Engl. J. Med. **350**, 1353-1356

4. Gerecht-Nir, S. and Itskovitz-Eldor, J. (2004) Best. Pract. Res. Clin. Obstet. Gynaecol. **18**, 843-852

5. Gribaldo, L., Alison, M., Andrews, P.W., Bremer, S., Donovan, P.J., Knaan-Shanzer, S., Mertelsmann, R., Spielmann, H., Testa, N.G., Triffitt, J.T., Zipori, D. and de Wynter, E. (2002) Exp. Hematol. **30**, 628-633

6. Rohwedel, J., Guan, K., Hegert, C. and Wobus, A.M. (2001) Toxicol. In. Vitro. **15**, 741-753

7. Schatten, G., Smith, J., Navara, C., Park, J.H. and Pedersen, R. (2005) Nat. Methods. **2**, 455-463

8. Mosmann, T. (1983) J. Immunol. Methods. **65**, 55-63

9. Ready, T. (2001) Nat. Med. **7**, 1262

10. Heng, B.C., Kuleshova, L.L., Bested, S.M., Liu, H. and Cao, T. (2005) Biotechnol. Appl. Biochem. **41**, 97-104

Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

List of abbreviations

bFGF : basic fibroblast growth factor

DMEM : Dubelcco's minimum essential medium

FCS : fetal calf serum

hESC : human embryonic stem cells

MEF : murine embryonic fibroblast

min : minute

MTT : tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBS : phosphate buffered saline

Table and figure legends

Table 1 MTT assay on hESC, their differentiated fibroblastic progenies and CRL-1486 (control) after treatment with the two different protocols, before and after freeze-thawing.

Figure 1 (A) before trypsinization, (B) after treatment with 0.05% trypsin for 2-3 min, prior to gentle pipetting.

Figure 2 Undifferentiated hESC (A) after collagenase and scratching, (B) exposure to MTT after collagenase and scratching, (C) after trypsinization and gentle pipetting, (D) exposure to MTT after trypsinization and gentle pipetting.

Figure 3 Differentiated hESC fibroblastic progeny at P3 (A) after collagenase and scratching, (B) exposure to MTT after collagenase and scratching, (C) after trypsinization and gentle pipetting, (D) exposure to MTT after trypsinization and gentle pipetting.

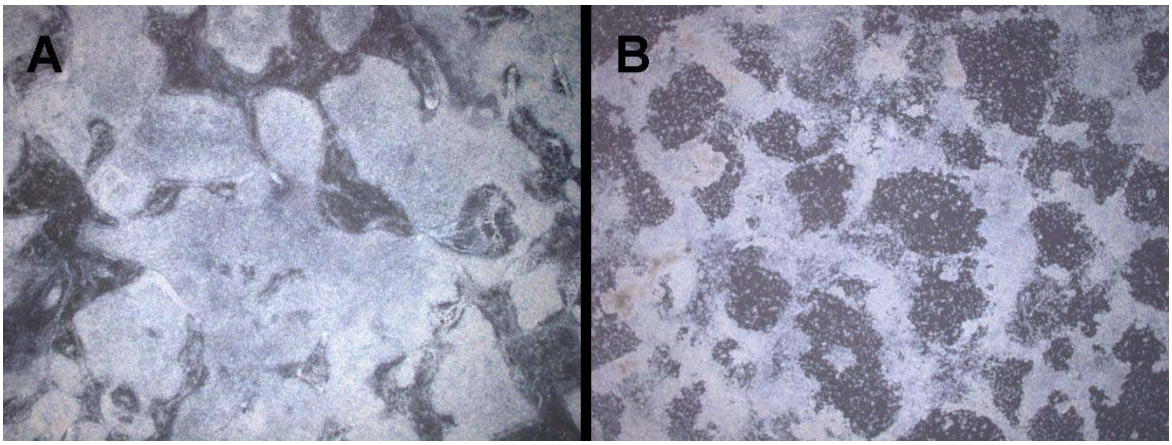
Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

Table 1

Cell type	Duration of random free differentiation (days)	Cell numbers	Protocol 1: Collagenase + scratching			Protocol 2: Trypsinization + pipetting			% recovery of viable cells in protocol 1 as fraction of protocol 2 (without freeze-thaw)
			Absorbance at 570 nm (after correction for blank)		Freeze-thaw survival rate	Absorbance at 570 nm (after correction for blank)		Freeze-thaw survival rate	
			Before freeze-thaw	After freeze-thaw		Before freeze-thaw	After freeze-thaw		
hESC	0	7.5×10^5	0.90 ± 0.01	0.26 ± 0.01	28.9 %	1.55 ± 0.01	1.09 ± 0.01	70.5 %	58.0 %
P0	7	4.5×10^5	0.45 ± 0.01	0.16 ± 0.01	35.9 %	1.96 ± 0.02	1.78 ± 0.01	90.8 %	22.9 %
P1	14	1.0×10^6	0.48 ± 0.02	0.27 ± 0.03	56.0 %	1.82 ± 0.03	1.71 ± 0.03	94.2 %	26.4 %
P2	21	8.0×10^4	0.09 ± 0.01	0.06 ± 0.01	73.7 %	0.36 ± 0.01	0.33 ± 0.01	92.2 %	25.9 %
P3	28	2.5×10^5	0.67 ± 0.02	0.50 ± 0.01	75.0 %	2.12 ± 0.02	1.86 ± 0.01	87.8 %	31.4 %
P4	35	7.0×10^5	0.44 ± 0.01	0.26 ± 0.01	59.5 %	1.87 ± 0.03	1.76 ± 0.05	94.0 %	23.7 %
CRL-1486	--	7.5×10^5	0.81 ± 0.01	0.74 ± 0.01	91.0 %	1.53 ± 0.02	1.42 ± 0.03	92.6 %	53.2 %

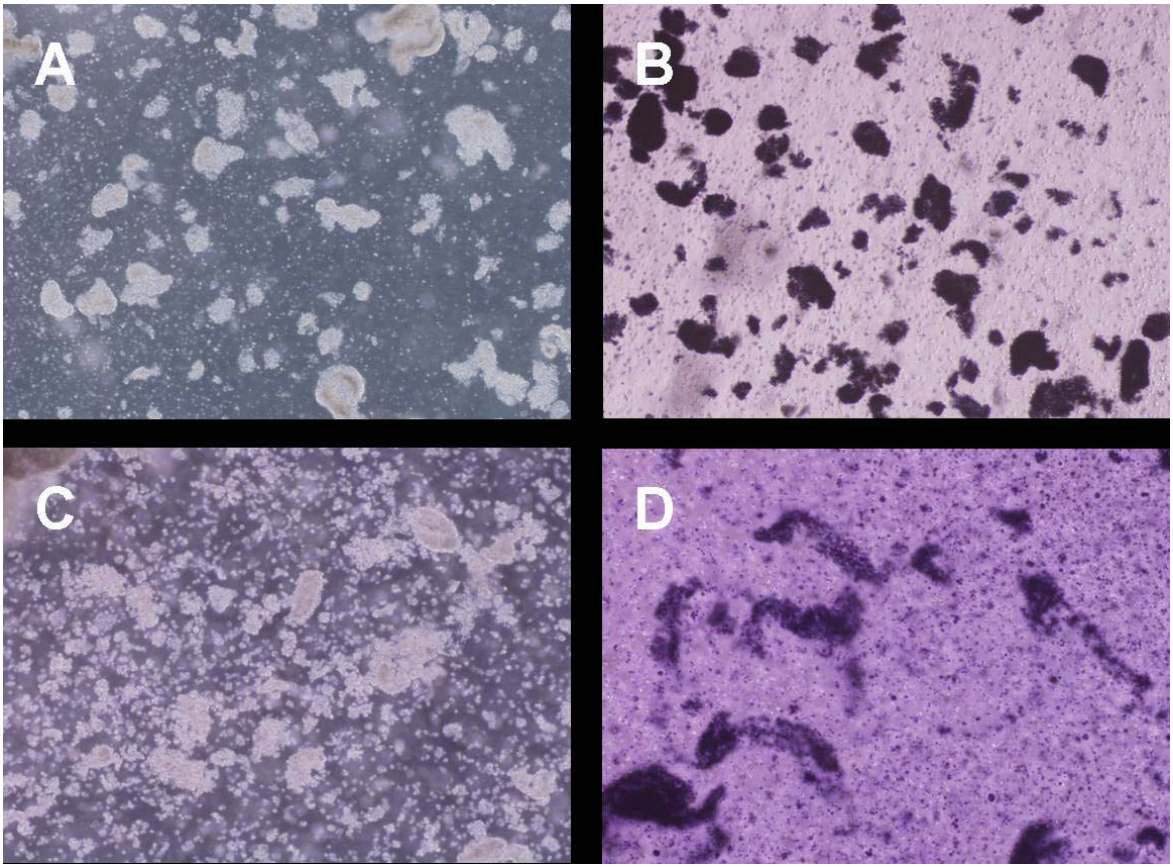
Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

Figure 1



Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

Figure 2



Biotechnol. & Appl. Biochem. Immediate Publication. Published on 21 Nov 2006 as manuscript BA20060151

Figure 3