PROTOCOLS FOR HUMAN PLURIPOTENT STEM CELL WORK

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GROWTH MEDIA

Scott Noggle, doc. version 6, 2-7-10

HUESM is the current growth media that we use for growing hESCs and iPSCs and differs from the standard H1 medium described by Thomson's group in the use of KO-DMEM rather than DMEM/F12. The original formulation of H1 medium called for 4ng/ml of bFGF. Current studies suggest that MEFs respond optimally to a higher concentration of bFGF. We have increased the bFGF concentration in HUESM (see table below) to reflect this. This dose of bFGF seems to compensate for some degree of variation between MEF batches. However, it is best to culture cell lines in bFGF conditions recommended by the supplier of the cell line.

HUESM	CATALOGUE NO.	FINAL CON.	FOR 500ML
Knockout-SR	10828	20%	100ml
GlutaMAX	35050	2mM	5ml
MEM non-essential amino acids	11140-050	0.1mM	5ml
Penicillin-streptomycin	15140-122	100U/ml-0.1mg/ml	5ml
2-Mercaptoethanol	21985-023	0.1mM	900ul
KO-DMEM	10829-018	to volume	385ml

KSR is thawed at 4oC, aliquoted into 50ml conical tubes and frozen at –20oC. A stock of growth medium is stored at 4^{o}C for no more than two weeks. Preheat only as much medium as is needed for ~ 20 to 40 min @37^{o}C. To prepare complete growth medium, bFGF is added just before feeding (see bottom table).

APPLICATION	BEFORE CONDITIONING ON MEFS	BEFORE FEEDING HESCS
Maintenance on MEF feeder layers	na	10ng/ml
Maintenance on Matrigel	20ng/ml	20ng/ml

Stocks of bFGF (Invitrogen cat 13256-029) are made to 100µg/ml in sterile 10mM Tris-HCl pH7.6/0.1%BSA. 20ul and 8ul aliquots are frozen at -20^{o}C and once thawed, are not refrozen.

DEFINED MEDIA: We are currently evaluating several defined media, including mTeSR1 (Stem Cell Technologies) and NeutriStem XF/FF (Stemgent).

MEF-CONDITIONED MEDIUM (CM)

Scott Noggle, doc. Version 1.5 2-5-10

For some studies, it may be important to culture HESCs or iPS cels in the absence of feeder cells. This protocol is provided for generating Mouse Embryonic Fibroblast (MEF) - conditioned medium for culture of HESCs or iPS cells on Matrigel coated dishes. commercially available Mitomycin-C treated MEFs of the CF-1 strain can be used. We have used MEFs from GlobalStem (GSC-6001G or GSC-6001M) and Specialty Media (PMEF-CF). Alternatively, MEF feeder cells from derived in house can be used. Primary MEFs are used between 1 to 5 passages. See accompanying protocol for isolation and inactivation procedures if you are producing you own MEFs.

Passaged HESCs are plated in 2ml of CM per well of a 6-well plate. 5ml on a 60mm dish, or 8ml on a 10cm dish. They can be fed with the same volume for the first few days. When the colonies get bigger, increase the CM to 3ml/7/12ml, respectively. Cultures in CM on Matrigel can usually grow for 5 days before they need passaging.

MATERIALS AND PREPARATION OF CONDITIONED MEDIUM:

Dishes: Coat 10cm dishes in 0.1% gelatin (made in TC-grade distilled water) for at least 20min in the incubator.

Medium:

FM10	CATALOGUE NO.	FINAL CON.	FOR 500ML
FBS		10%	50ml
GlutaMAX	35050	2mM	5ml
Penicillin-streptomycin	15140-122	100U/ml-0.1mg/ml	5ml
2-Mercaptoethanol	21985-023	0.1mM	900ul
DMEM (high glucose)	10829-018	to volume	439ml

Plating MEFs: Thaw one vial of GlobalStem or Specialty Media Mitomycin-C inactivated MEFs (5x106 cells) or equivalent inactivated MEFs and resuspend in 12ml of FM10 medium. Aspirate the gelatin from the plates and immediately plate the 12ml of cells directly on one gelatin coated 10cm dish. Incubate overnight to attach.

Conditioning medium: The next day, rinse the MEFs with HUESM and replace with 12ml of HUESM with bFGF. Incubate overnight to condition the medium. After 24hrs, draw off the conditioned medium into a 50ml tube and replace with fresh HUESM. I try to keep to 24hrs of conditioning (+/- 2-4hrs is ok). The CM can be used immediately, stored at 4oC for a week or frozen at –80oC. When ready to use to feed Matrigel cultures, add fresh FGF2 before plating see table below for concentrations of bFGF.

APPLICATION	BEFORE CONDITIONING ON MEFS	BEFORE FEEDING HESCS
Maintenance on Matrigel	20ng/ml	20ng/ml

PRODUCING MEF FEEDER CELLS

Scott Noggle, doc. Version 1.3 2-5-10

We are currently using commercially available Mitomycin-C treated Mouse Embryonic Fibroblast (MEF) of the CF-1 strain from GlobalStem (GSC-6001G or GSC-6001M). Alternatively, feeder cells can be prepared from E13 ICR embryos (strain CD-1 from Charles River Laboratory) and inactivated using Mitomycin-C or gamma irradiation. Primary MEFs are used between 1 to 5 passages. This protocol is used for producing MEFs in house.

Medium: FM10: DMEM containing 10%FBS, 1X Lglutamine, 1X pen-strep, and 100μM 2-Mercaptoethanol (all from Gibco).

Isolation of p0 MEFs from embryos: Alternatively, primary Mouse Embryo Fibroblasts are isolated from E13 embryos essentially as described in Manipulating the Mouse Embyro[1]. Briefly, sacrifice a pregnant mouse by an institutionally approved method. Swab the mouse liberally with 70% ethanol. Using scissors make a cut across the belly and cut away the skin to expose the gut. With sterile forceps and scissors, dissect out the uterus and place it into a Petri dish in sterile PBS. Isolate the embryos from the uterus, and release the embryos from the embryonic membranes. Transfer embryos to a second Petri dish with sterile PBS. Using watchmaker forceps under a stereomicroscope, remove the embryo heads and liver, intestines, heart and all viscera and gonadal ridges with two pairs of watchmaker forceps leaving only the limbs and body cavity. Transfer the cleaned embryos into a sterile 10mL syringe with 5mls of 0.25%Trypsin/EDTA per 10 embryos. Pass the embryos and trypsin through an 18G needle slowly and gently, into a fresh Petri dish. Collect the partially dissociated embryos and trypsin with a serological pipette and pass through the needle a second time. Incubate the tissue for 15 minutes at 37oC, pipetting the tissue a few times through a 10ml pipette to dissociate the tissue. Allow the large pieces of cellular debris to settle (5 minutes 1g). Remove the supernatant into a fresh tube and add about an equal volume of fibroblast medium. Spin down cells and resuspend in medium. Discard the debris. One embryo is plated on a T175 flask in a total of 30 mls of medium per flask. This density allows the cells to adhere but not become overly confluent before harvest at Day 3-4. Incubate at 37oC with 5% CO2. MEFs will attach and begin to divide overnight. Change the medium every other day. When the flasks are nearly confluent, usually in 3-4 days, the cultures are ready for freezing. Freeze cells in 10% DMSO/90%FBS at 6x106 or 12x106 per vial. This is considered passage p0. It is also a good idea to screen MEF batches for mycoplasma.

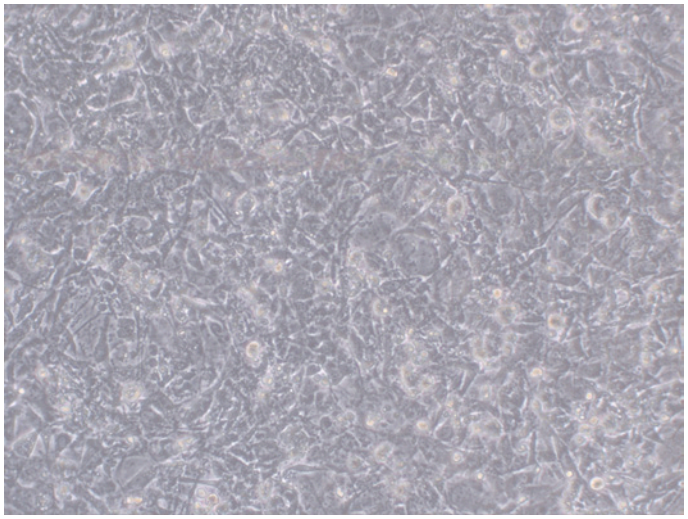
Inactivation by Mitomycin-C: Inactivated MEFs are prepared by thawing p0 MEFs. Cells are thawed quickly in a 37oC water bath with gentle shaking. The cells are gently transferred to a 50 ml conical tube with 20mls of fibroblast medium and centrifuged at 200xg for 4 minutes. The cells are resuspended in 30-50 mls medium and counted with trypan. Viability should be >95%. Cells in fibroblast medium are seeded into T175 flasks at 1.8X106 per flask with 25 mls medium per flask. Flasks are incubated at 37oC 5% CO2. This cell number should be optimized to give 75-90% confluency by day 3. On day 2, aspirate medium and add 35 mls fresh fibroblast medium to each T175. On day 3 after thawing, cells should be 75-90% confluent in T175 flasks. Check each flask to assure proper cell growth and sterility. Medium is aspirated and cells are treated with mitomycin-C at a final concentration of 10μg/ml in15 mls per T175 fibroblast medium for 2.5 hours at 37 oC and 5% CO2. To prepare mitomycin-C, dissolve 2 mgs per vial powdered mitomycin c (Sigma, Cat#M4287) in 200 mls fibroblast medium. This working stock is 10μg/ml. It can be stored at 4oC protected from light for up to 6 weeks or frozen at -20_ for longer storage. After use, add 15 ml bleach per 500 mls mitomycin-C soln to neutralize it.

After incubation, the mitomycin-C is aspirated and cells are washed with 20 mls PBS. Aspirate the PBS and add 15 mls fibroblast medium to each flask. It is convenient to only trypsinize five to six flasks at a time to minimize exposure to the trypsin. Start by rinsing the flasks an additional 3 times by rinsing once with 20 mls PBS and twice with 15 mls each of Ca-Mg-

free-PBS. After the last wash is aspirated add 2ml of 0.05% trypsin/EDTA. Disperse the trypsin by tilting the flask. Dissociation of the cell layer typically takes 1-2 minutes. When the cells detach from the flask 5 mls of fibroblast medium are added to each flask to stop the trypsin. Pool cell suspensions from the flasks in a 50 ml conical tubes and bring to 50 mls with fibroblast medium. Wash the cells once with 25ml of fibroblast medium. Count the cells and access viability with trypan blue. Plate as described below. Alternatively, the inactivated MEFs can be frozen as above and thawed for subsequent use. Freeze cells in 10% DMSO/90%FBS at $5x10^6$ per vial.

Inactivation by gamma-irradiation: MEFs to be inactivated are harvested as above, combined into one 50ml tube and irradiated with a dose of 5000 to 8000RADs. The time of exposure must be calculated based on the activity of your gamma source. Optimal dose should be determined by plating irradiated cells at clonal density and monitoring for colony growth. Pick the lowest dose that yields little colony formation. Count the cells and access viability with trypan blue. Plate as described below. Alternatively, the inactivated MEFs can be frozen as above and thawed for subsequent use. Freeze cells in 10% DMSO/90%FBS at $5x10^6$ per vial.

Plating inactivated MEFs for HESC or iPS culture:



Dishes are pre-coated with 0.1% gelatin made with cell culture grade water for 20-30min in the incubator. Inactivated MEFs are plated fresh from the inactivation procedures outlined above or thawed from commercially available vials. If using previously frozen vials, MEFS are resuspended in the appropriate amount of medium and plated directly - they are not to be centrifuged. A density of $0.3X10^5/cm^2$ is a good starting density for MEF plating. This value should be optimized to give complete coverage without being too dense (see photo to right), as increased MEFs in the culture will deplete culture media components. Conversely, too few MEFs will not provide adequate conditioning of the media. Plating density is shown in the table below. Inactivated MEF feeder cells should be plated a day or two before additions of HESCs to allow for attachment and spreading of the MEF layer to completely cover the surface of the dish. It is important that the HESCs not come in contact with tissue culture plastic before the MEFs have laid down sufficient ECM to prevent premature differentiation. 35mm tissue culture treated dishes are most convenient, however larger dishes also work well. MEFS are fed every other day until used. MEF feeder layers should not be used after 4 days.

VESSEL	SURFACE AREA (PER WELL)	NUMBER OF MEFS (PER WELL)	OPTIMUM VOLUME
35mm dish	$10cm^2$	$0.3\text{-}0.5x10^6$	2ml
60mm dish	$20cm^2$	$0.5\text{-}1x10^6$	5ml
T25 flask	$25cm^2$	$0.75\text{-}1.2x10^5$	5ml
4-well plate	$2cm^2$	$0.5x10^5$	750µl

MATRIGEL PLATE COATING

Scott Noggle, doc. Version 1.2 9-28-07

GENERATING MATRIGEL STOCKS:

The Matrigel that we are currently using is qualified by Stem Cell Technologies to maintain hESCs. Thaw one 5ml vial of Matrigel (BD cat# 354277) at 4oC overnight. The original Matrigel stocks came at different stock concentrations and were diluted to a final of 0.333mg/ml to coat plates. This usually meant roughly a 1:30 final dilution. The current stocks do not come with a concentration listed, but instead come with dilution instructions. It is a good idea to check the Product specification sheet for the dilution factor for the current lot of Matrigel. If these are not available, call BD at the phone number listed on the vial and give them the lot number. They will tell you or email the sheet for that lot. It is important to keep the vial and all pipettes and tubes ice cold to prevent premature gelling of the matrix. Using the cold pipette, dispense the 10ml vial into 9-10 1ml aliquots in pre-chilled cryotubes on ice. These can be refrozen at –20oC.

COATING PLATES:

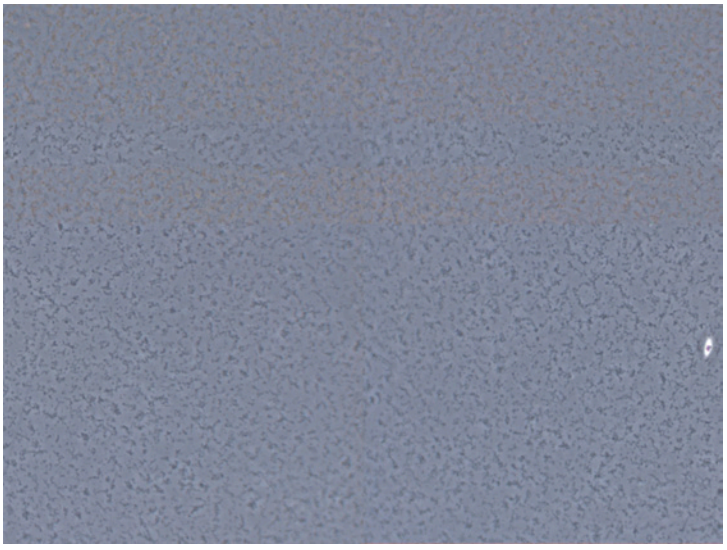
[Instructions for the current lot - you may need to change the dilution factors] In preparation, place a 512ul aliquot (from above or thawed slowly on ice, about 2hours) on ice. Prepare an ice bucket with a metal support tray for the plates/dishes. We use shallow rectangular ice buckets filled with ice and with small metal incubator trays on top of the ice. Pre-cool 6-well plates or dishes. Pre-cool p1000 filter-tips at –20oC for about 20min.

Dilute the 512ul aliquot of Matrigel with cold base medium (XVIVO-10, DMEM/F12, DMEM) to 40ml total in a pre-cooled 50ml conical tube. Mix well and dispense 2ml into each well of the 6-well plates or 8ml onto a 10cm dish (equivalent amounts per surface area of other dishes). Keep the plates on the ice cold platform at all times. If in a rush the plates can be kept at RT for about 4hrs in a tissue culture hood (covered with foil) to coat. Some lots of Matrigel will tolerate this and some will not. Alternatively, place the entire ice bucket with plates into the refrigerator to coat overnight. Sometimes, the plates will coat better after two days in the refrigerator.

PREPARATION OF COATED PLATES FOR HESCS:

30-2hr min before passaging, warm the plates with Matrigel in the incubator to allow the Matrigel to gel.

Before plating cells, check the coating on the microscope for a meshwork-like single layer matrix (see photo to right). When ready to plate HESCs, aspirate the Matrigel from the wells using a Pasteur pipette in the corner of the well. Get as much Matrigel off of the dish as possible leaving a thin coating on the surface of the dish. Do not scrape the bottom of the dish. Rinsing the Matrigel-coated plate is not necessary. Plate HESCs in conditioned medium or other desired conditions.



FIBROBLAST CULTURES FROM SKIN BIOPSIES

Gist Croft (modified by Florian Merkle)
1-29-09
modified by Scott Noggle 2-7-10

Purpose: This protocol is used to establish fibroblast cultures from human skin biopsies. The whole procedure usually takes ~4 weeks: 1-3 weeks for fibroblasts to expand to occupy most of the area underneath the coverslips, then another ~1 week for Passage 1 fibroblasts to cover a T25 or T75 flask.

MATERIALS:

A. Equipment

- Laminar Flow Hood
- Water Bath, 37oC
- Pipet-Aid
- Incubator, 5% CO2
- Tabletop Centrifuge
- Hemacytometer with cover glass
- Microscope, inverted
- Ice buckets with lids
- Dry Ice
- Ice
- Label maker

B. Supplies

- Plastic Serological Pipets: 1, 2, 5, and 10 ml
- Latex gloves
- Sterile straight and curved micro-forceps
- Sterile, disposable scalpels
- Spray Bottle, 70% Ethanol
- Tissue culture treated 60mm plates
- T25 Tissue Culture Treated Flask
- Acid-washed Microscope Cover Slips (soak overnight in 10% HCL and rinse with distilled water)
- Autoclaved silicone grease
- Sterile 200ul pipet tips
- 15ml conical tubes
- Freezing Vials, 1.8ml

MEDIA AND CHEMICALS:

MATERIALS	VENDOR	CATALOG NUMBER
Freezing Media 1X	Millipore	ES-002-10F
Nucleosides	Millipore	ES-008-D
PBS	Fisher	MT21040CV
Trypsin-EDTA	Invitrogen	25300-112
Coverslips	Corning	2865-22
Silicon Grease	Dow Corning	2021854-0499
Parker super O lube	Silicon base	347306

BIOPSY PLATING MEDIA	CATALOGUE NO.	FINAL CON.	FOR 500ML
FBS		10%	50ml
GlutaMAX	35050	2mM	5ml
MEM non-essential amino acids	11140-050	0.1mM	5ml
Antibiotic-antimycotic	15240-062	1X	5ml
2-Mercaptoethanol	21985-023	0.1mM	900ul
Nucleosides	Millipore		5ml
KO-DMEM	10829-018	to volume	425ml

BIOPSY CULTURE MEDIA	CATALOGUE NO.	FINAL CON.	FOR 500ML
FBS		10%	50ml
GlutaMAX	35050	2mM	5ml
Penicillin-streptomycin	15140-122	100U/ml-0.1mg/ml	5ml
KO-DMEM	10829-018	to volume	440ml

Media and Buffer preparation: Media and Buffers must be prepared under sterile/aseptic conditions and filter sterilized. Sanitize all exterior services of bottles and tubes prior to entry into laminar flow hood.

PROCEDURE

On the Day of Patient Biopsy:

a. arrangements are made for picking up biopsy on retrieval day
b. Biopsy samples are stored in the Biopsy Plating Media (SOP #5000) at 4oC until ready for pick-up

Plating Biopsy Samples:

a. Carefully wipe all tubes with 70% Ethanol, make note of Subject ID, especially if there are multiple tubes.
b. Set-up 4-5 60mm Petri dishes for each biopsy sample. [Note: Use 2 square coverslips (each with several small biopsy chunks under it, held down by its own dab of silicone grease) per petri dish, rather than one coverslip per petri dish. Petri dishes and flasks are standard TC treated from NUNC or BD]
c. Carefully extract the biopsy punch from the conical tube with 1 ml pipet.
d. Wash the biopsies several times in PBS +Penn/Strep and pace inside a 60mm Petri dish. [Note: biopsy is fairly sticky, especially on dry plastic, and chunks will stick stubbornly if aspirated into serological pipets]
e. Carefully hold the biopsy punch with a pair of curved forceps and with a sterile scalpel, carefully mince the biopsy core into 10 pieces.
f. With a pair of straight-edge forceps, place 2-3 pieces of minced biopsy core on a new 60mm dish.
g. Using a sterile 200ul pipet tip, scoop out a little of the autoclaved silicone grease and dab next to the minced core pieces. Using a sterile forcep, take an acid-washed coverslip and place over the grease and minced biopsy pieces. Press firmly down on coverslip.
h. Add 5mls of biopsy plating media and place into incubator. Do not disturb for five days.

Culturing and Freezing Fibroblast Cells:

a. After five (5) days, replace spent media with culture media.
b. Small outgrowths of cells should be visible at day 5. Replace spent culture media with fresh culture media, every 3-4 days, until the coverslip is confluent.
c. Cells are ready to be split when the entire coverslip is covered with fibroblast cell growths. This may take 2-3 weeks. Cells grow mostly on plastic.
d. Pre-warm trypsin, prior to use. Wipe carefully with 70% Ethanol before placing inside laminar flow hood. Gelatinize a T25 flask fro every 60mm petri dish.
e. Remove spent media and wash the plate surface with PBS. With a 200ul pipet, carefully pry the coverslip from the Petri dish bottom and overturn inside the Petri dish. The cell growth surface should be facing up. Add 3ml of pre-warmed trypsin and replace into incubator for 5 minutes. Make sure the overturn coverslip is covered with trypsin.
f. Check trypsin digestion every 5 minutes by removing the petri dish and observing under microscope. When the cells are no longer attached to the coverslip, harvest the cells into a 15ml conical tube. Inactivate trypsin with 1:1 volume of fetal bovine serum, or culture media (contains 10% FBS).
g. Spin at 500x g for 5 minutes. Wash once with 5mls of media and spin at 500x g for 5 minutes.
h. After wash, aspirate media and re-suspend in 5mls. Remove gelatin water from T25 and add cell suspension to flask.
i. Feed T25s every 2-3 days until confluent, this may take 1-2 weeks. When cells are confluent, harvest and freeze cells. Freeze 3 vials per T25 flask. [Note Cryopreservation of cells is same as for primary mouse embryonic fibroblasts. We've originally used the flash freeze on dry ice method, but we now use the traditional 1 degree/minute slow freeze isopropanol chambers.]

IPSC INDUCTION PROTOCOLS - RETROVIRUS

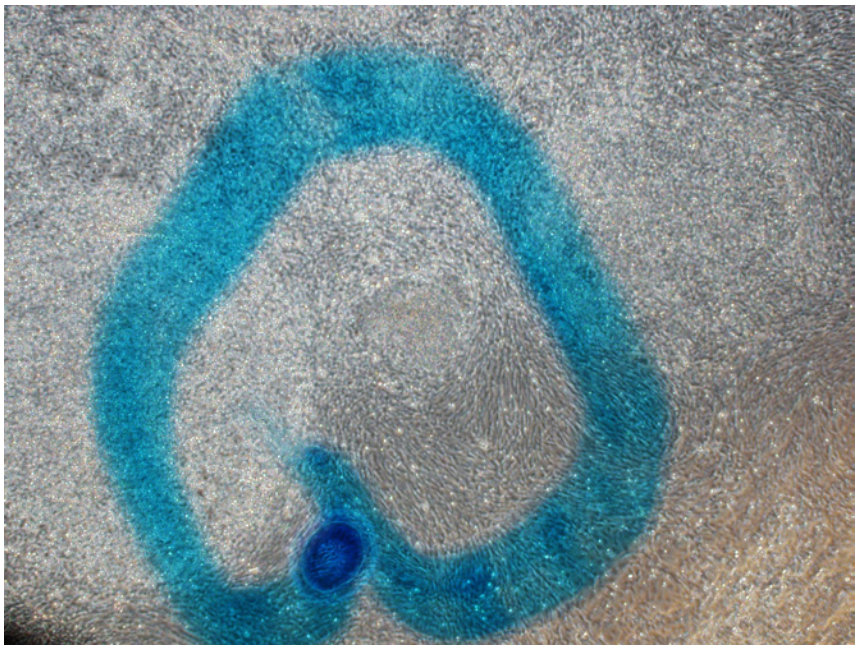
HUMAN IPS METHODS

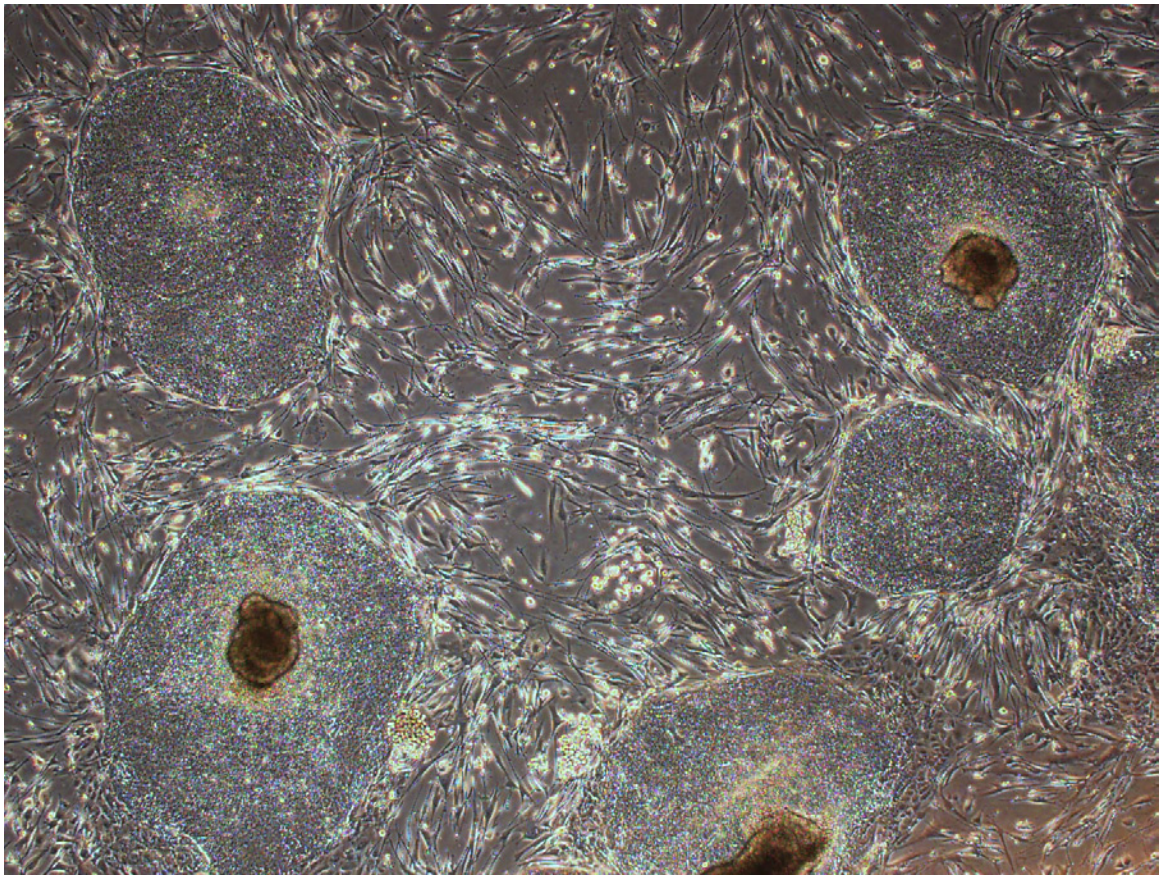
Dieter Egli, doc version 1.0 2-5-10

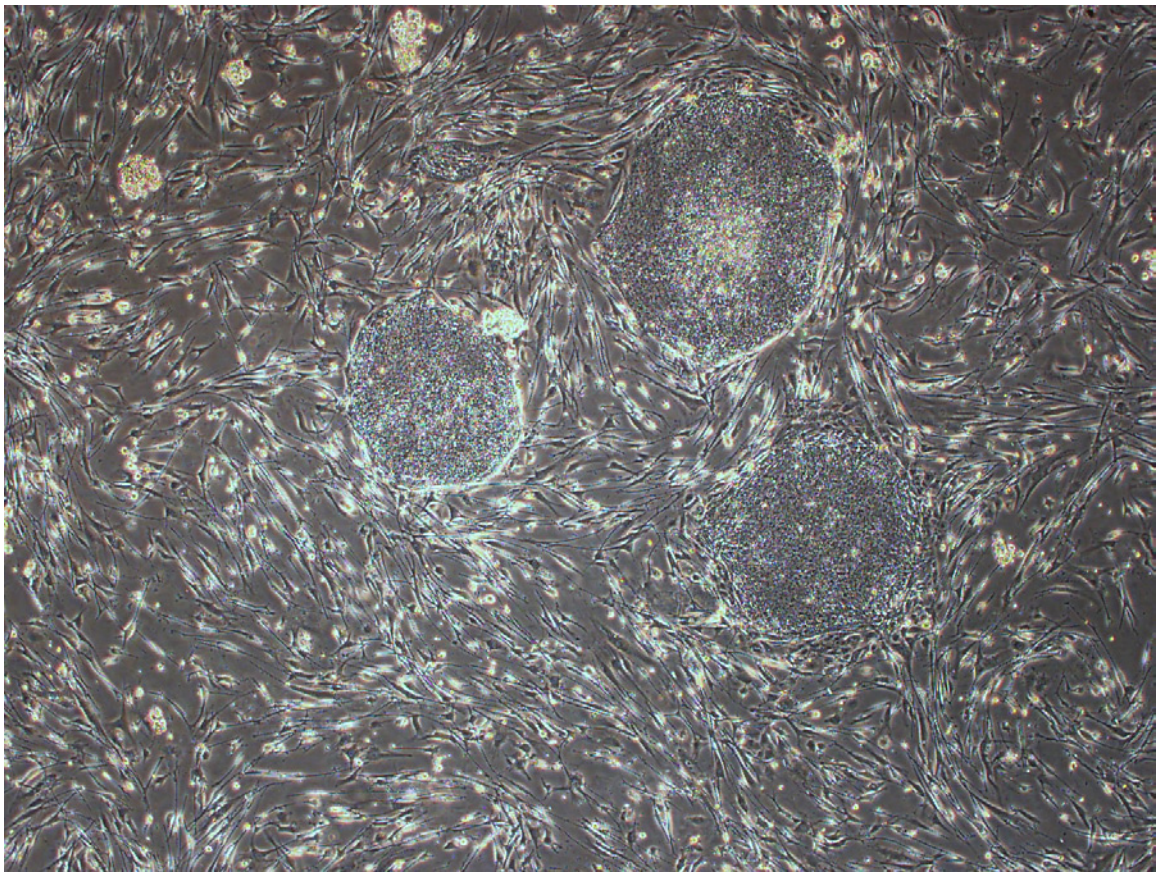
The protocols for inducing bona fide iPS cells are still evolving. This protocol represents our current best practices for generating iPS cells. Our gold standard system for generating iPS cells relies on retroviral introduction of the factors identified by Yamanaka to reprogram adult fibroblasts. The original retroviruses have been modified for higher expression of the factors Oct4, Sox2, Klf4 and optionally c-myc. As a high rate of infection and high initial expression of the factors correlates with successful reprogramming, we currently purchase high-titer retroviral stocks based on these modified retrovirus vectors from the Harvard Gene Therapy Core Facility. The following is a brief description of how these high titer stocks were produced.

Retroviral production. Human cDNAs for KLF4, SOX2, OCT4, and CMYC (OpenBiosystems) were sub-cloned into the murine leukemia viral vector pMXs-Tcl1 (Addgene plasmid 13364) (S2, S3). Moloney gag-pol (pUMVC; Addgene plasmid 8449) and VSV-g envelope (pCMV-VSV-g; Addgene plasmid 8454) (S4) were obtained from Addgene. These plasmids were transiently co-transfected into 293FT packaging cells (ATCC) at a 10:9:1 ratio (transgene:gag-pol:VSV-g) using SuperFect (Qiagen). Viral supernatant fractions were harvested after 60 hours, filtered through a 0.45 μm low protein binding cellulose acetate filter, and concentrated by centrifugation.

iPS generation. To produce patient specific iPS (PS-iPS) cells, we are currently infecting 10,000 patient fibroblasts in a 6-well dish. The infections are in 1ml of HUESM with another 1ml of fibroblast medium added one day later. The cultures are fed every day with a half change of HUESM. When cultures become confluent, HUESM medium is exchanged every day with 3ml of media. It is important not to let media turn yellow. After eight to nine days, cells are passaged with TrypLE at a density of 20-30,000 cells per well of a 6-well dish coated with MEFs. Medium is replaced with HUESM medium the following day, and cells are subsequently cultured in standard HUESM medium until colonies appear. Colonies are circled (right) and remaining cells, including MEFS, are aspirated or scraped away under a dissection scope. Fresh MEFs are plated on top of the remaining colonies. Continue feeding with HUESM and repeat cleaning of cultures to prevent overgrowth of non-iPS cells, usually after three to four days. When iPS colonies are large, they are manually picked and passaged with a cell scraper or pipet (see protocol for colony picking). This is repeated for approximately 1 month before adaptation to enzymatic passage with trypsin and subsequent characterization. Colonies below are examples of an iPS line after three pick passages.







MOUSE IPS METHODS

Justin Ichida, doc. version 1.0 6-16-09

Derivation of MEFs and cell culture: MEFs were derived from E12.5 embryos hemizygous for the Oct4::GFP transgenic allele. Gonads and internal organs were removed before processing the embryos for MEF isolation. MEFs were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. Low passage (up to passage 3) MEFs were used for generation of iPS cells.

Retroviral production and infection: Moloney-based retroviral vectors (pMXs) expressing the murine complementary DNAs of Oct4, Sox2, c-Myc, and Klf4 were obtained from Addgene. These plasmids were transfected separately into individual populations of Plat-E packaging cells using Fugene 6, with 27 μl of Fugene 6 and 9 μg of DNA per 10cm dish of Plat-E cells. Viral supernatants were obtained 48- 72 hours post-transfection, filtered through a .22 mm filter, diluted 1:1 in MEF growth media, and supplemented with polybrene at a final concentration of 5 mg/ml. The supernatants for the four factors were mixed in an equimolar ratio, and media was used in place of a factor when it was omitted from the infection. MEFs were infected with two to three pools of viral supernatant during a 72-hour period. The first day that viral supernatant was termed “day 1 post-infection.”

The following protocol was adapted from Takahashi et al. Induction of pluripotent stem cells from fibroblast cultures. Nature protocols (2007) vol. 2 (12) pp. 3081-9.

Thawing and passage of Plat-E cells: Prepare 9 ml of FM10 medium in a 15-ml tube. Remove a vial of frozen Plat-E stocks from the liquid nitrogen tank and put the vial in a 37 1C water bath until most (but not all) cells are thawed. Aseptically transfer the cell suspension to the tube. Centrifuge at 180g for 5 min, and then discard the supernatant. Resuspend the cells with 10 ml of FM10 medium, and transfer to a gelatin-coated 100-mm dish. Incubate the cells in a 37 oC, 5% CO2 incubator. Replace the medium 24hrs later with new media supplemented with 1 μg/ml of puromycin and 10 μg/ml of blastcidin S. Continue to incubate the cells in a 37 oC, 5% CO2 incubator until they are 80–90% confluent. Passage with 0.05% trypsin/0.53 mM EDTA, at 1:4–1:6 dilution to 100cm plates with antibiotics. Cells should become confluent within 2–3 d.

Retrovirus production: Passage cells with 0.05% trypsin/0.53 mM EDTA. Count the number of cells and adjust the concentration to $8x10^5$ cells per ml with FM10 medium. Seed cells at $8x10^6$ cells (10 ml) per 100cm culture dish, and incubate overnight.

The next day prepare for transfection into Plat-E cells: Transfer 0.3 ml of DMEM into a 1.5-ml tube. Deliver 27 μl of Fugene 6 transfection reagent into the prepared tube, mix gently by finger tapping and incubate for 5 min at room temperature. Add 9 μg of pMXs plasmid DNA (encoding Oct3/4, Sox2, Klf4 and c-Myc) drop-by-drop into the Fugene 6/DMEM-containing tube, mix gently by finger tapping and incubate for 15 min. Add the DNA/Fugene 6 complex dropwise into the Plat-E dish, and incubate overnight at 37 1C, 5% CO2. The next day, aspirate the transfection reagent–containing medium, add 10 ml of fresh FM10 medium, and return the cells to the incubator. Collect the medium from the Plat-E dish 48-72 hrs later by using a 10-ml sterile disposable syringe, filtering it through a 0.22 μm pore size cellulose acetate filter, and transferring into a 15-ml tube. Dilute 1:1 with fresh FM10 medium and supplement to 5 μg/ml polybrene. The supernatants for the four factors were mixed in an equimolar ratio and MEFs were infected with two to three pools of viral supernatant during a 72-hour period. Media was replaced with mouse ES media supplemented with Lif.

Generation of iPS cells

GFP+ P0 colonies were picked manually and incubated in .25% trypsin (Gibco) for 20 minutes at room temperature before plating on a feeder layer in mES cell media. This process was repeated until passage 3, at which time colonies were trypsinized and passaged in bulk and maintained on feeders in mES cell media.

IPS COLONY PICKING

Scott Noggle, doc. versiton 1.3 3-3-10

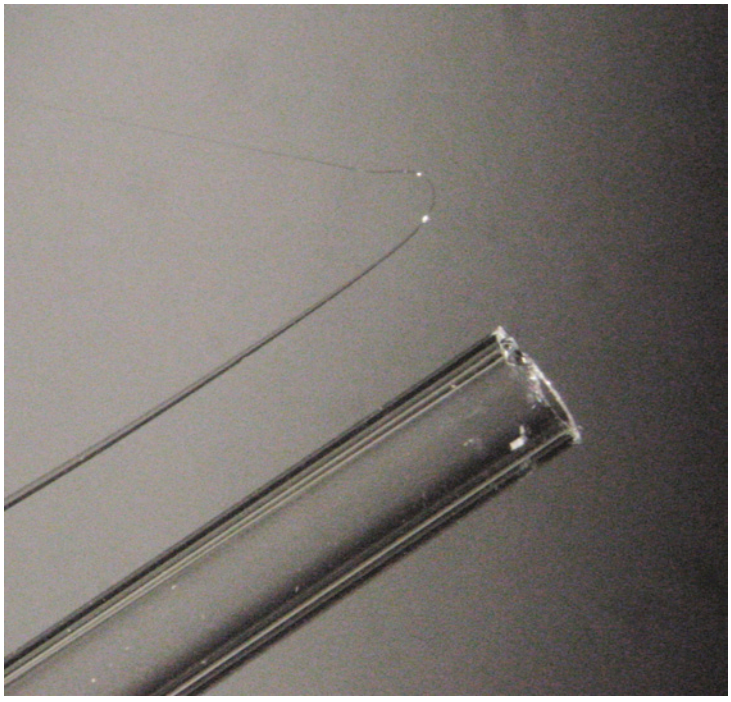
MICRO-DISSECTION METHOD FOR PICKING HESCS AND IPSC COLONIES

Scott Noggle, doc. version 1.5 2-7-10

Purpose: Used for initial colony picking of iPSCs and to maintain master stocks of HESCs and iPSCs.

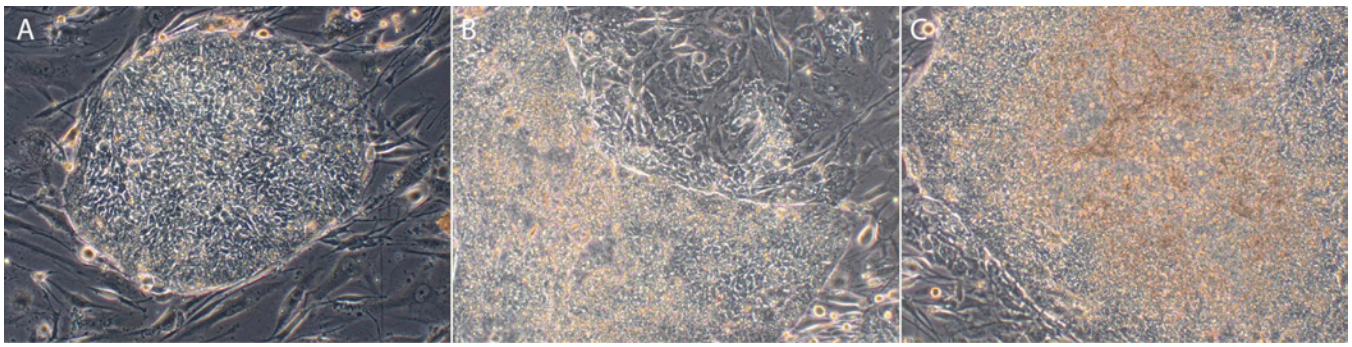
Feeder cells: See accompanying protocol for generating MEF-feeder plates.

Tools: Glass tools or syringe needles can be used for micro-dissection of colonies for passaging. Additionally, it is also possible to pick iPS colonies with a pipette tip or cell lifter. However, for fine control of dissection when initially isolating good areas of iPS colonies (or HESCs) from undesirable regions of the colony, glass tools are optimal tools. For glass tools, Pasteur pipettes are pulled hair thin. Fine glass needles with hooked ends are forged in two steps over a microburner assembled as described in Manipulating the Mouse Embyro[1] as follows. While holding the two ends of a long Pasteur pipette, place the thin end of the pipette at a distance of about half to two-thirds of the distance away from the tip into the orange part of the flame until the glass melts into a solid constriction. In a single motion upon removing the pipette from the flame, pull on each end of the pipette gently and quickly to draw out a thin filament before the glass hardens. This is done without breaking the connection between the two ends of the pipet. Second, beginning several inches above the flame and slowly moving the thin drawn part of the filament down towards the flame pull a very fine filament as previously described. The two ends of the pipette should separate, this time forming a fine needle end on the tip of the pipette. If the tip remained straight after the second pull, pass the fine end a few inches quickly over the top of the flame. The force of the rising heat will curl the tip of the needle into a hook. The hooked end should be thin enough for the micro-dissection of the colonies but thick enough to withstand some pressure during the dissection. Examples of the final product are shown in panel A of the figure below.



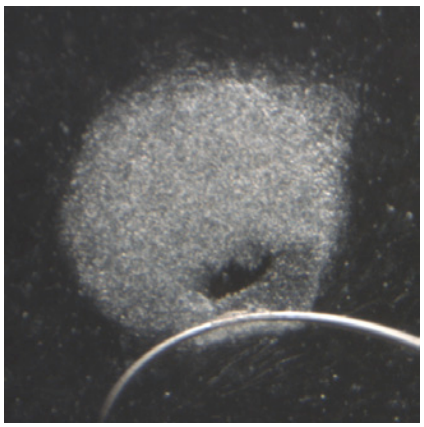
Observation of cultures to be passaged: For iPS cell colony picking from the original induction, you must first identify HESC-like colonies from a mix of partially reprogramed and transformed colonies. Some examples of good and bad iPS colonies are shown in the sections following this protocol. These protocols can also be used to passage master stocks of iPS cells or HESCs growing on feeders. In these instances, before passaging, examine the colonies under the microscope and choose colonies that are undifferentiated. Avoid colonies or parts of colonies that are showing signs of differentiation. Several types of differentiation can be morphologically identified in spontaneously differentiating cultures. Avoid the center of colonies

that show a depression or “crater” appearance. Areas of colonies that have begun forming cystic structures should be avoided. Also avoid the edges of colonies that do not have a tight border between the feeder layer and the colony. In these areas, the HESCs/iPSCs have started to flatten, polarize and migrate into the feeder layer. Some differentiation on the borders of the colonies can be tolerated, as these cells can be left behind with the micro-dissection technique. In some cases, it may be necessary to dissect a colony that has begun to differentiate. The region of differentiation can be avoided selecting only the undifferentiated parts of the colony to dissect. Leave the differentiated regions untouched. Ideal colonies are comprised of small, round, and randomly organized cells with a high nuclear to cytoplasmic ratio that have not begun forming structures within the colony (examples are shown in panel A in the figure below).



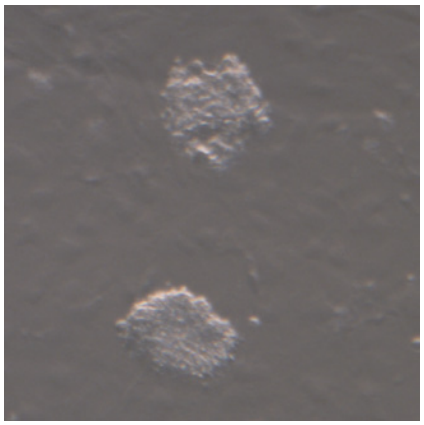
Preparation for passaging: Prior to micro-dissection, medium is changed in the well to be passaged and on the feeders. The feeders are washed once and 2ml of fresh complete growth medium is placed on the new feeders on 35mm dishes. Care is taken to maintain temperature, pH and osmolarity of the media by working quickly. The feeders are kept in the incubator during the dissection. Alternatively, a humidified CO_2/O_2 mixed gas source and a warm plate are convenient to maintain an optimal environment while the cultures are out of the incubator. This is especially helpful after the colonies are transferred to the new feeders, as it is important to minimize handling of the dish. If the dishes are handled too much before the colonies have attached to the feeders, the chunks of HESCs will migrate to the center of the dish and attach too close together.

Passaging: Ideal colonies or parts of colonies are micro-dissected into chunks of about 100 cells using the glass hooks. The hook is used to gently pull apart pieces of the colony (see photo to right). This can also be accomplished by cutting a grid into the colony with the back of the hook and pulling the pieces away from the colony one at a time. It is easier to pull out one piece at a time, as large pieces are more difficult to cut into smaller pieces. The size of the piece should be large enough to survive the cutting and adhere to the feeder layer (see photo on next page). A piece too large will tend to form an embryoid body-like structure on the feeder layer as it takes too long for the entirety of a large colony to come into contact with the feeders. The resulting colony will have an area of differentiation in the center arising from the embryoid body-like structure (see panel C in the figure above for an example).



After micro-dissection, the cell chunks are swirled into the center of the dish and 20 to 50 chunks are transferred to the new feeder wells using 1ml micro-pipets. Pre-coat the micro-pipet tip with the medium so that the cells do not stick (a regular sterile pipet or Pasteur pipet can be used also). Transfer no more than 500ul of medium to the new dish. In some cases, it may be necessary to transfer the entire well volume to the new feeder well or wells. Exchange medium 2.5 to 3ml/well. If possible, leave the dishes untouched on a warmed

surface (preferably under O2/CO2 blood-gas mix) for 15-30min to allow the chunks to begin attaching to the dish before moving to an incubator. Excessive handling of the new dish will cause the chunks to migrate to the center of the dish rather than remaining evenly distributed across the dish. Good spacing between the colonies will allow proper growth of the colonies.

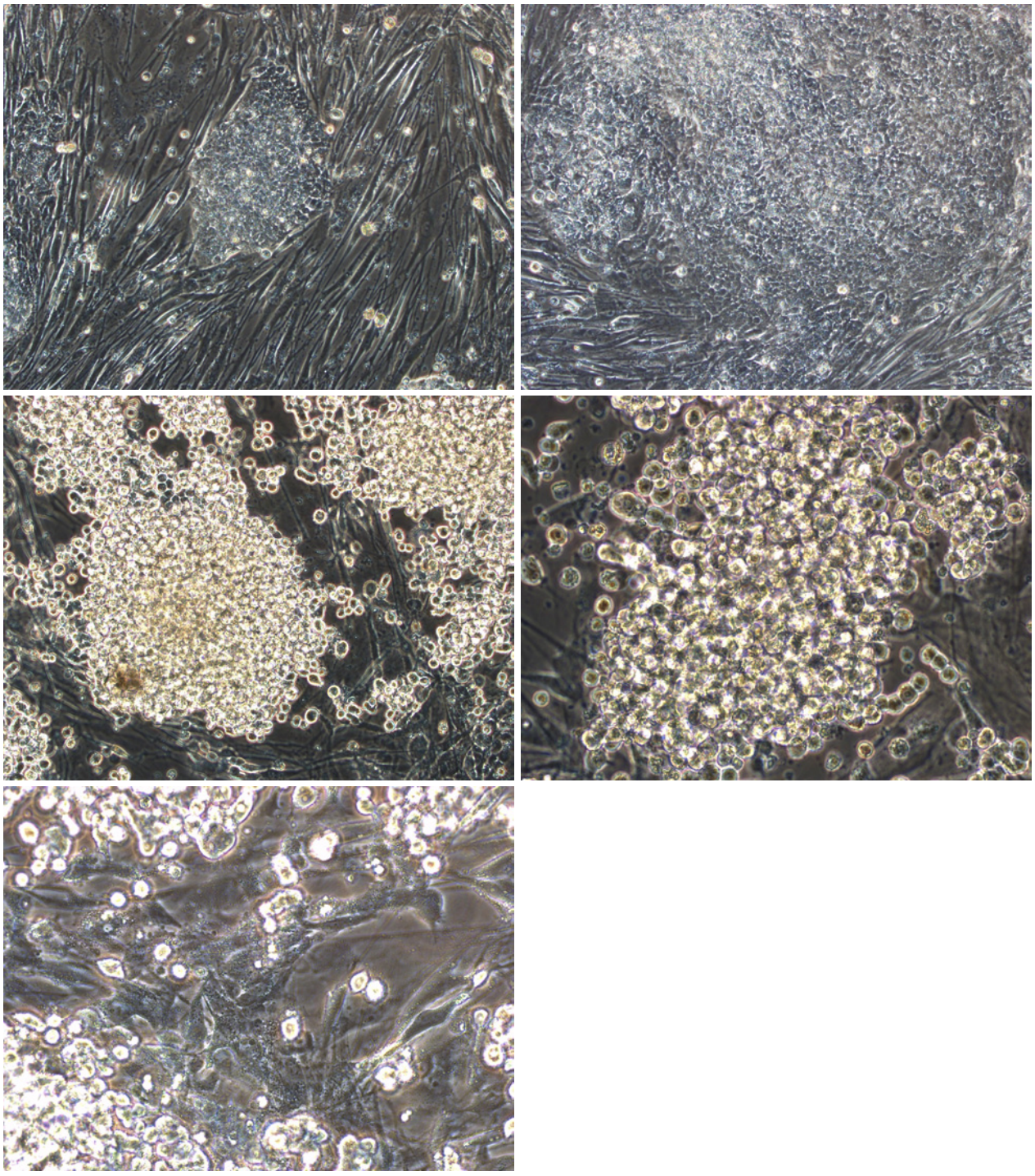


Maintenance: Complete growth medium is exchanged on the growing colonies every day as the feeder layer can use up nutrients quickly. In the example photos BG01 cells are used. The cell cycle for this line is about 24-36 hours. The lines should culture for no more than 6 days to a week. The timing of passage is dependent upon the appearance of differentiation within the colonies—mainly from the center and edges of the colony (see figure above for examples of differentiation).

References:

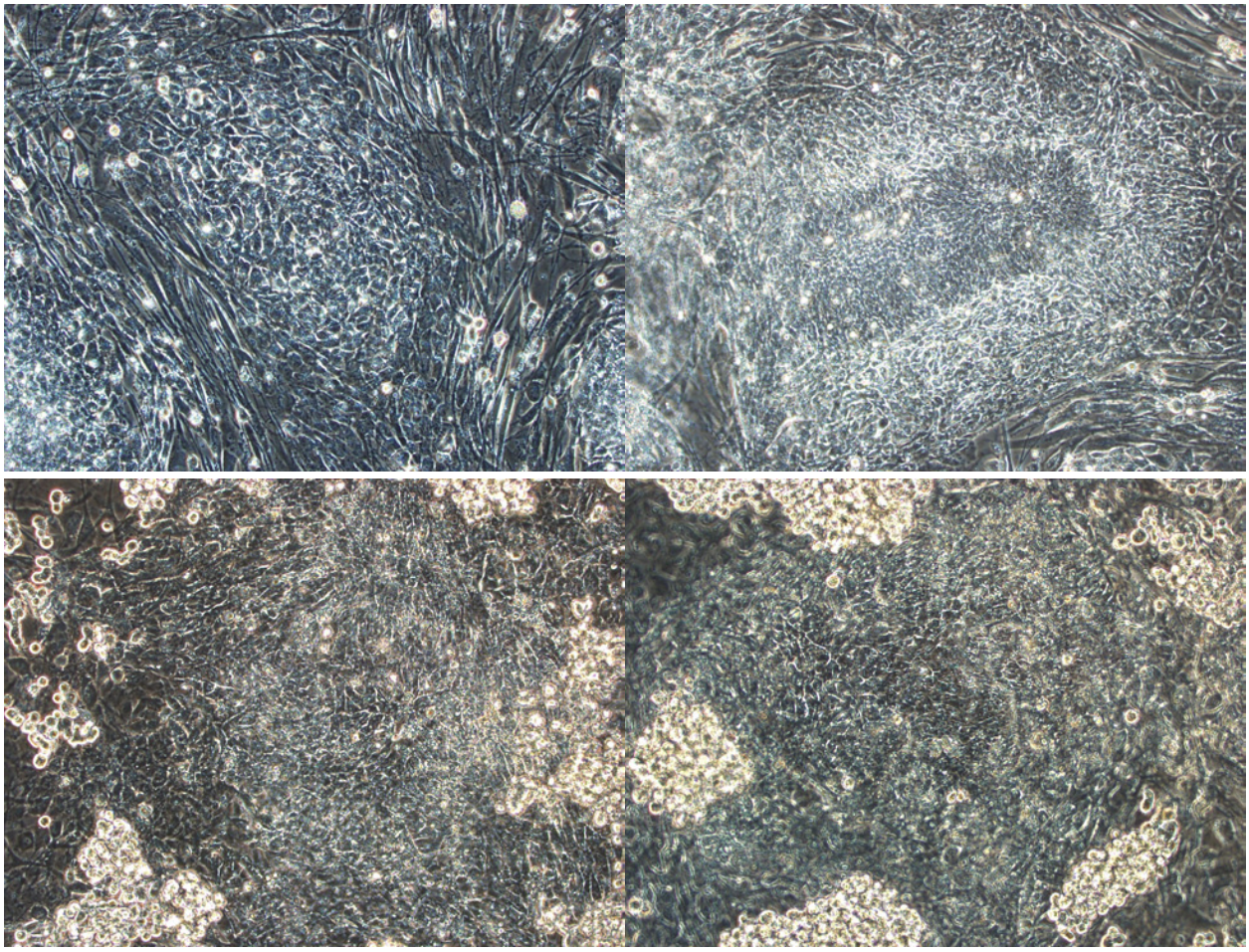
1. Hogan, B. Manipulating the mouse embryo: a laboratory manual (Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1994).

EXAMPLES OF BAD IPS CELL COLONIES



These are examples of partially reprogrammed or transformed colonies. These can be recognized by lack of HESC morphological appearance(eg no bright borders between cells) or as small, phase-bright loosely adherent cells.

EXAMPLES OF GOOD IPS CELL COLONIES



These are examples of good iPS colonies in initial phases of induction. The top two photos are reasonably free of transformed cells (phase-bright, loosely adherent) and are composed of colonies with HESC morphology (eg bright borders between individual cells within the colony, large nuceli, large nucleus). The bottom two photos have good adherent colonies surrounded by transformed cells. It is possible to carefully clean the colony of the transformed cells before passaging the good colonies.

PASSAGING METHODS FOR HESC AND IPSC LINES

THE EZPASSAGE TOOL

Dieter Egli, doc version 1.0 2-5-10

Purpose: Used to expand established and homogenous cultures and master stocks of HESCs or iPSCs.

Passaging: When colony density has reached confluence in the dish or become too numerous to manually passage, an alternate protocol is to use a serrated roller tool from Invitrogen to cut the colonies into small uniform sized pieces for passaging. Media is exchanged and the tool is used to roll over colonies several time in one direction, then several times with the plate rotated 90^{o}. The pieces are gently triterated with a pipette and plated onto fresh plates.

Reusing tools: The tools can be cleaned by soaking in 70%ETOH for 1hr, then rinsing in sterile H2O. They are placed in a dry beaker and UV treated overnight.

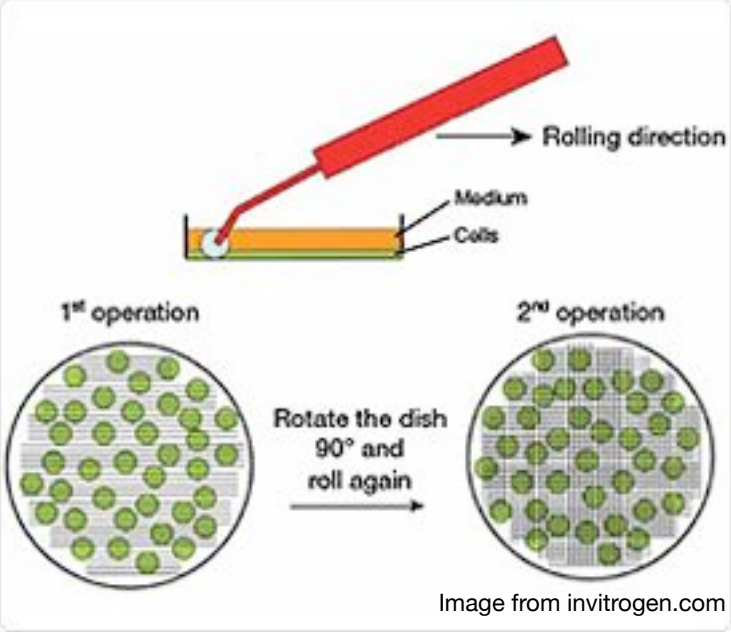


Image from invitrogen.com

ENZYMATIC PASSAGING OF HESCS AND IPS CELLS

Scott Noggle, doc. Version 1.4 9-28-07

Purpose: For expansion of HESCs on Matrigel (or other matrix) coated plates in MEF-CM but can be modified for HESCs grown directly on MEFs.

MATERIALS AND PREPARATION:

Feeder cells for MEF-CM: See accompanying protocol for generating MEF-conditioned medium.

Matrix: Tissue culture plates are coated with Matrigel as described in the accompanying Matrigel plate coating protocol.

Medium: Growth medium is described in the section on Growth medium for HESCs and the section on generating Conditioned Medium. A stock of growth medium is stored at 4oC for no more than one week. Preheat only as much medium as is needed for ~ 20 to 40 min @37oC.
Enzyme: Dispase or Collagenase, type V (either at approximately 1mg/ml) dissolved in growth medium (and sterile filtered). We are currently buying Dispase from Stem Cell Technologies. Dilute these stocks 1:5 in DMEM or DMEM/F12.

TRANSFERRING HESCS:

Before passaging, examine the colonies under the microscope and look for any colonies that are differentiated. Spontaneously differentiating areas of the culture can be removed with a glass tool as described in the manual dissection protocol or aspirated using a pipette attached to a vacuum. Several types of differentiation can be morphologically identified in spontaneously differentiating cultures. Look for the center of colonies that show a depression or “crater” appearance. Areas of colonies that have begun forming cystic structures in the center of the colony should be removed. Also avoid the edges of colonies that do not have a tight border between the feeder layer and the colony. In these areas, the HESCs/iPSCs have started to flatten, polarize and migrate can also be removed. However, some differentiation on the borders of the colonies can be tolerated, as these cells will detach during the washing steps (see below). Ideal colonies are comprised of small, round, and randomly organized cells with a high nuclear to cytoplasmic ratio that have not begun forming structures within the colony (examples are similar to those shown in the manual dissection protocol).

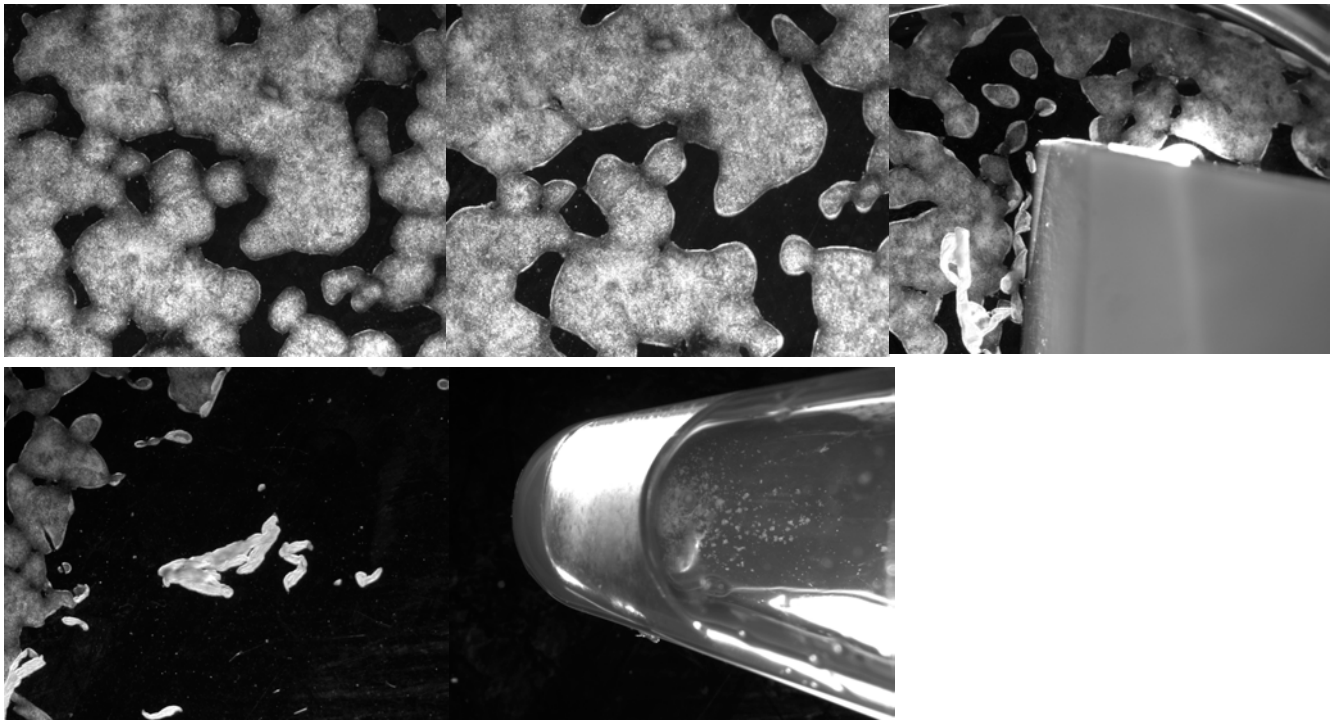
Passaging: Replace the growth medium with Dispase or Collagenase. Allow to incubate in the incubator for about 7 min. Check the progress of the matrix digestion, beginning at about 4 min. The colony borders will begin to peel away from the plate, while the center will remain attached. Ideally, gently wash the Dispase or Collagenase off of the plate with growth medium twice. The colonies should remain attached to the plate. If they have detached after the Dispase incubation transfer all of the colonies and Dispase solution to a conical tube and centrifuge and wash the colonies with growth medium. They should get two to three washes total – either on the plate or with centrifugation. If the colonies remained attached after washing, harvest the colonies with a cell lifter (Costar 3008 – NOTE: DO NOT USE THE SWIVEL-HEAD CELL SCRAPER (3010)). Transfer all of the colonies and growth medium to a conical tube and spin to pellet the colonies. Using the MEF-CM, resuspend the colonies using a p1000 pipette tip in about 500-700ul of medium. Triterate the colonies to clumps with an

average size of about 100 cells using the p1000 tip. Plate a proportion of the clumps (I currently use a 1:10 split ratio – but you will need to adjust this for the confluency of the starting population).

If possible, leave the dishes untouched on a warmed surface for 10 min. to allow the chunks to begin attaching to the dish before moving to an incubator. Excessive handling of the new dish will cause the chunks to migrate to the center of the dish rather than remaining evenly distributed across the dish. Good spacing between the colonies will allow proper growth of the colonies.

Maintenance: Complete conditioned growth medium is exchanged on the growing colonies every day from the MEF plates. The lines should be cultured for no more than 6 days before passage. The timing of passage is dependent upon the appearance of differentiation within the colonies—mainly from the center or outer edges of the colony.

Cryopreservation and recovery: See accompanying protocol for cryopreservation in tubes or straws.



This sequence shows (from top left to bottom right) colonies before dispase treatment, after treatment, during scraping, after scraping and after triteration in a 15ml conical tube.

FREEZING IPS CELLS

Faizzan Ahmad, doc. version 1.3 3-3-10

We currently freeze iPS lines using standard slow cooling in the presence of 10% DMSO. However, there are some modifications to the traditional procedure that improve viability of the iPS cells. If you are having difficulty with viability, an alternate protocol is included for vitrification of colonies. We freeze one confluent well of a 6-well plate into 3 vials at early passages. This can be increased at later passages when iPS cell cultures have stabilized.

MATERIALS:

DMSO [Sigma cat. D2650] - IMPORTANT NOT TO LET STOCKS GET OLD
Growth Medium (see section on Growth medium for HESCs)
FBS
Cryovials
Nalgene Cryo 1°C Freezing container

Freezing media:
40% Growth medium (eg. HUESM)
50% FBS
10% DMSO
Prepare all fresh, sterile filter, and maintain on ice while working.

For thawing, prepare MEF coated plates according to MEF plating protocol.
6-well plates

FREEZING PROTOCOL:

1. Chill all solutions and tubes on ice and place Nalgene Cryo container at -80°C to begin cooling.
2. Passage cells using either the EZpassage tool or by enzymatic passaging (Trypsin or Collagenase)
3. pellet and resuspend clumps in 0.5ml of cold Freezing media.
4. transfer to cryotubes on ice.
5. transfer to Nalgene Cryo container at -80oC
6. freeze overnight then transfer to LN2.

THAWING PROTOCOL:

1. On the day prior to thawing, plate MEFs onto 6-well plates as described in the MEF plating protocol.
2. Warm growth media at room temperature before starting the thaw.
3. Remove vials from LN2.
4. Thaw quickly in a 37oC water bath until only a small ice pellet remains.
5. add 1.5ml of growth media slowly to the cryovial to dilute the cryoprotectant.
6. Transfer the 2ml of cells and growth media from the cryovial to a 15ml conical tube.
7. Add growth media to 10ml.
8. spin at 800 for 5-7 min.
9. resuspend in 2ml of growth media (with bFGF)
10. plate onto 1 well of a 6-well plate.

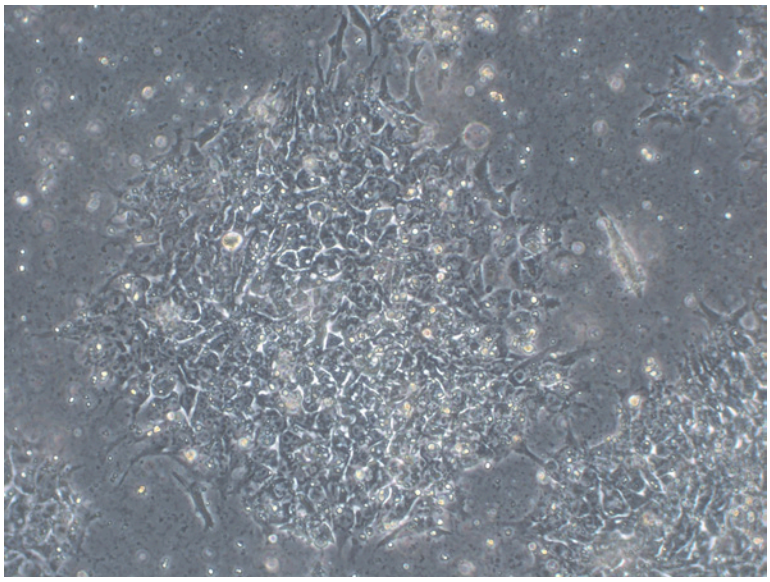
ALTERNATE PROTOCOL: FREEZING BY VITRIFICATION IN CRYOVIALS

Scott Noggle, doc. Version 1.2 1-30-07

We have had difficulty cryopreserving RUES1 hES cells using traditional means of freezing hES cells. Survival is typically less than 1%. To improve recovery, we have optimized a protocol based on vitrification (Richards et al., 2004). This protocol uses cryovials instead of straws as originally described. This allows for higher through-put and faster processing times during the procedure. We have typically seen 40-50% recovery using this protocol. (The photo below is RUES2 on the day after thawing.)

MATERIALS:

1. Ethylene glycol [Sigma cat E9129]
2. DMSO [Sigma cat. D2650]
3. Growth Medium (see section on Growth medium for HESCs)
4. Sucrose [Fisher cat S5-500]
5. 1M HEPES solution [Invitrogen, cat 15630-080]
6. Cryovials
7. liquid nitrogen in an ice bucket
8. Square floating microtube rack [Nalgene 5974-0404]



PREPARE MEDIA:

HM (Growth Medium with 20mM HEPES):
80% Growth medium (eg. HUESM)
20mM HEPES

HM+Sucrose:
3.42g Sucrose in 10ml HM

VS2:
30% HM
30% HM+Sucrose
20% ethylene glycol
20% DMSO

VS3:
40% HM+Sucrose
30% ethylene glycol
30% DMSO

WS3:
Growth medium +1M Sucrose

Prepare all fresh, sterile filter, and maintain on ice while working.

FREEZING PROTOCOL:

Note: Work quickly. The HESC cannot be exposed to the cryoprotectants for very long or they will differentiate upon thawing. Viability may also be reduced if timing is not closely controlled. Steps 4-11 must be timed accurately.

1. Harvest HESCs in clumps by manual dissection or collagenase/dispase treatment. This protocol can also be used on collagenase/dispase-harvested HESC grown on Matrigel or MEFs.
2. Wash clumps well to remove collagenase/dispase if necessary.
3. Resuspend clumps into HM in about 0.5ml (depending on number of clumps). They can be kept at room temp in HM for up to 20min. Prolonged incubation will result in clumping and reduced attachment after thawing.
4. Transfer 40ul of the clumps into a sterile cryovial on ice in the microtube rack. Process 5 vials at a time.
5. Add 40ul of VS2 and mix by gentle pipetting let sit a 10-20 seconds
6. Add 160ul of VS3 and mix by gentle pipetting. Steps 5-6 should be completed in no more than 1 min. Handle only as many tubes as can be processed in this amount of time. Remember that handling and capping the tubes will take time.
7. Submerge the tubes quickly in liquid nitrogen and swirl while freezing. The frozen solution should have a pink glass-like appearance, while a thin layer at the top might be opaque. Be sure the caps are tightened and transfer vials to liquid nitrogen storage boxes. It is important to do this quickly to prevent the small solution volume from thawing. I usually place a storage box in 1-2 inches of LN2 in a large rectangular ice bucket while I am processing and transferring the tubes.

THAWING PROTOCOL:

Thawing is performed in the tubes and all solutions must be prepared in advance. Steps 1-3 must be performed quickly so that the cells are not exposed to the high concentration of cryoprotectants for too long. The incubation times in steps 4-7 remove the sucrose slowly and prevent osmotic shock and lysis of the cells.

1. Remove a tube from liquid nitrogen storage and quickly submerge bottom of tube in warm sterile water in a beaker.
2. quickly wipe with 70%ETOH-soaked kim-wipe.
3. Immediately add 800ul of cold WS3 (growth medium + sucrose), mix gently by stirring with the pipette tip and let sit for 30 seconds.
4. Add 1ml of growth medium, mix as above, let sit for 2min.
5. transfer to 15ml conical tube.
6. Rinse cryotube 2 times with 1ml each of growth medium and add to 15ml tube, mixing gently, let sit for 1min.
7. Add 6ml growth medium slowly dropwise to cells over about 2 min.
8. Spin 1000/4min
9. Resuspend gently in 1ml of growth medium with p1000.
11. Using a p1000 to transfer to a well of a 6-well plate with MEFs in growth medium. I have also thawed directly onto Matrigel with success. The colonies should recover and show signs of growth within a week. Change medium daily.

KARYOTYPING

Scott Noggle, doc. versiton 1.2 2-11-10

We currently send out iPS cultures to a commercial service for karyotyping (Cell Line Genetics). However, depending on your access to in house services, you may need to provide fixed cells for analysis. This protocol was communicated to me by Maya Mitilipova, who used it to generate samples of BG01 and BG02 for karyotype analysis by G-banding. I have used it to generate samples of RUES1 and RUES2 for karyotype analysis by the cytogenetics service at Sloan Kettering. Alternatively, some services like to have cell actively growing. Check with your service to be sure they are comfortable and have experience handling human ES cells or iPSCs.

PROTOCOL:

1. Feed the cells the day before karyotyping
2. Add colcemid for two hours (10ug/ml stock), 20ul per 1.5ml of medium.
3. Collect the supernatant into 15ml conical tubes and trypsinize cells, break into single cells and collect into the same tubes.
4. Centrigfuge cells at 1000rpm
5. Add about 2ml warm KCL (0.56% or 0.075M) and incubate at 37 for 20 min
6. Add 6-8 drops of fresh 3:1 (methanol:acetic acid) fixative and incubate for additional 15 min at room temp.
7. Centrifuge for 8min at 1000rpm
8. Remove supernatant and add 2ml of 3:1 fixative and incubate at RT for 10 min
9. Repeat step 7
10. Remove supernatant and add 2:1 fixative and refrigerate overnight at 4oC
11. Next morning change fixative and drop slides to check for proportion of metaphase spreads
12. Store at -20oC
13. send off for G-banding.

TERATOMA ASSAYS

Scott Noggle, Taken from chapter in Human Embryonic Stem Cells; The Practical Handbook. 9-28-07

The ability for hES cells to generate teratomas (Keller G, 2005; Spagnoli FM and AH, 2006) in immuno-compromised mice is used as a diagnostic criteria for bona fide embryonic stem cells. In this in vivo assay, hES cells are engrafted into immuno-compromised adult mice in various tissues to generate teratomas. The resulting tumors are routinely analyzed by histology for the various derivatives of the three primary germ layers. With the exception of the host vasculature within the tumor, the teratomas are predominantly derived from the hES graft (Gertow et al., 2004). In the case of the vasculature, it was noted that both human graft-derived cells and host derived mouse cells can contribute to the vessel structures. Frequently, other differentiated and organized tissue can be found in the tumors. This can include, for example, neural tissue and retinal pigmented epithelium, muscle, cartilage, bone, and epithelial cells of the endoderm and ectoderm. However, many of these tissues may be immature and definitive identification of the mature tissue can be difficult. The assistance of a trained pathologist in evaluating the tissues is highly recommended.

Teratoma can be generated at various sites in adult SCID mice by subcutaneous, intraperitoneal or intramuscular injection, implantation under the kidney capsule or beneath the testis capsule (Pera et al., 2003; Przyborski, 2005). As the site of implantation may also influence the growth and differentiation of the teratoma (Przyborski, 2005; Cooke et al., 2006), it is recommended that several sites be tested to access the developmental potential of the hES cells. The strain of SCID mice may also make a difference in the success of engraftment (Przyborski, 2005). NOD-SCID mice are probably the best recipients, followed by the SCID-beige strain. Two protocols for implantation of hES cells into immuno-compromised mice are provided below. The protocols for subcutaneous, intraperitoneal, and intramuscular injection are similar and have the advantage of being technically simple to perform and do not require surgical manipulation of the mice. Alternative protocols for teratoma formation can be found in: Sullivan et al. Human Embryonic Stem Cells: The Practical Handbook - Page 123. (2007) pp. 404.

PROCEDURES FOR ENGRAFTMENT OF HES CELLS IN SCID MICE:

1. hES cells are harvested as for passaging as described in the procedures for preparing cells for microinjection or aggregation with approximately 100-200 cells per clump. The hES cells are suspended in a small volume of media (100ul per injection) and mixed with an equal volume of thawed Matrigel and transfer to cold cryotubes. The mixture is held on ice until loaded into the syringe just before injecting.
2. The hES cells are loaded into syringes fitted with a large gage needle. Load the cells into the syringe by drawing in a small amount of media followed by the hES cell suspension before attaching the needle.
3. The suspension is injected at subcutaneous, intraperitoneal or intramuscular sites:
    - For subcutaneous injection, target the needle beneath the skin on the rear flank.
    - For intraperitoneal injections, target the abdomen.
    - For intramuscular injection, penetrate the muscle of a single rear leg to minimize discomfort and alteration of the mobility of the mouse.
4. Monitor the mice and the site of injection weekly for 6-22 weeks. The mice should be weighed weekly and watched for signs of infection during the incubation period.
5. Teratomas can be recovered by dissection with surrounding tissue and usually arise between 6-8 weeks after grafting. They are fixed in formalin and sent for histological examination by a pathology service. Alternatively, they can be embedded for cryosectioning and processed for immunohistochemical detection of germ layer markers.

EMBRYOID BODY ASSAYS

Scott Noggle, doc. versiton 1.1 9-28-07

Embryoid bodies are formed by aggregating or placing clumps of HESCs in suspension culture such that they differentiate into the three primary germ layers.

GENERAL PROTOCOL:

1. Dispase treat cells as for 20 min until colonies are released from Matrix, whether on MEFs of MG.
2. Rinse colonies at least 2 times in media to remove dispase. Try not to break up colonies - they must remain in large chunks.
3. the cell clumps can be plated on bacterial dishes as a group or, alternatively, individually transferred to 96-well non-tissue culture treated V-bottom plates. Initial plating media can be MEF-CM or HUESM, then changed to serum containing media or specialty media. If EBs attach to the bacterial dishes, the dishes can be coated in agarose.
    1. Make a 1% stock of agarose in PBS.
    2. Autoclave to sterilize
    3. while hot, pipette 10ml onto a bacterial dish to cover the bottom of the dish, then immediately aspirate as much of the agarose as possible to leave a thin coating on the dish.
    4. Allow agarose to set up for 5-10 min at room temperature.
    5. Rinse once in media
    6. plate EBs in media
4. Culture for desired number of days. Change media every 2-4 days depending on the density of the EBs in the dish. Media changes in bulk culture are performed as follows:
    1. Using a 25ml pipette, transfer EBs to a 50ml conical tube.
    2. allow EBs to settle by gravity (5-10min).
    3. remove all but 5ml of media, being sure not to aspirate any EBs.
    4. Add fresh media and transfer to a bacterial dish (preferably agarose coated). It is not necessary to change dishes at every media change - usually every other media change.

IMMUNOFLUORESCENT PROCEDURES & MARKERS

These general protocols can be used for cells in culture on Matek coverslip plates, clear-bottom dishes for imaging or on mouse embryos and chimeras. If doing stains on embryos, filter the solutions to prevent particulates from sticking to the embryos. This will result in cleaner images. Embryos can be stained by transfering the embryos from solution to solution in 4-well or 24-well plates using a mouth pipetting apparatus. It is important to transfer only small volumes or rinse the embryo into the next solution. For the final step, embryos can be plated on Matek coverslips in the absence of protein to allow them to stick to the plate. This will prevent some migration of the embryos around the dish while imaging.

GENERAL PROTOCOL:

1. Wash plates once in 1X PBS
2. Fix in about 2ml of 4% PFA for 20 min at RTs
3. Wash two times in 1X PBS. The dishes can be stored at 4oC if sealed with parafilm
4. Block with 3% normal serum (species dictated by host of secondary antibody, usually Donkey or Goat for Alexa-conjugated secondaries) with 0.1% Triton-X in 1X PBS (without Ca/Mg) for 30 min at RT.
5. Remove Block and Add primary antibodies in Block
6. Incubate at 4oC overnight.
7. Wash three time for at least 30min each wash in PBST (PBS with 0.1% Tween-20) at RT. Washes can go overnight.
8. Add secondary diluted in Block
9. incubate at RT for 30min or overnight at 4oC
10. Wash twice in PBST for 30 min each wash
11. If counterstaining with SytoxOrange (or other nuclear counterstain), add in PBST. SytoxOrange is diluted at 1:25,000.
12. Wash twice in PBST and leave in PBST. They can be stored at 4oC sealed with parafilm or imaged. If photobleaching is a problem, the PBST can be removed and a drop of Vectashield mounting medium added. A coverslip can be placed on top to prevent evaporation.

MARKER ANTIBODIES:

	VENDOR	CAT. NO.	ISOTYPE	DILUTION	OTHER CONDITIONS
Pluripotency:					
Oct3	BD Transduction Laboratories	611203	Mouse IgG	1:500	0.1%Triton in Block
Oct4 (H134)	Santa Cruz	sc-9081	Rabbit poly	1:200	0.1%Triton in Block
Nanog	R&D Systems	AF1997	Goat poly	1:50	0.1%Triton in Block
Sox2	R&D Systems	245610	Mouse IgG	1:50	0.1%Triton in Block
SSEA4	Chemicon	MAB4304	Mouse IgG	1:100	no Triton
SSEA3	DSHB or Chemicon	MAB4303	Rat IgM	1:100	no Triton
Ectoderm:					
NFH	Sternberger	SMI32	Mouse, IgG	1:500	0.1% Triton

	VENDOR	CAT. NO.	ISOTYPE	DILUTION	OTHER CONDITIONS
beta III Tubulin (Tuj1)	R&D Systems	MAB1195	Mouse, IgG	1:75	0.1% Triton
Cytokeratin (Pan)	DAKO	Z0622	Rabbit poly	1:100	0.1% Triton
Cytokeratin 8	DAKO	M0631	Mouse IgM	1:50	0.1% Triton
Mesoderm:					
Muscle Actin (MF20)	DSHB	MF20	Mouse, IgG	1:10	0.1% Triton
Desmin	Abcam	ab8592-500	Rabbit Poly	1:100	0.1% Triton
CD31 (PECAM)	Abcam	ab9498-500	Mouse, IgG	1:100	0.1% Triton
Brachyury	Santa Cruz	sc-20109	Rabbit poly	1:50	0.1% Triton
Cardiac Actin	Fitzgerald	RD1-PRO61075	Mouse, IgG	1:10	0.1% Triton
Endoderm:					
AFP	DAKO	A0502	Rabbit poly	1:100	0.1% Triton
HNF3b	Santa Cruz	sc-6554	Goat poly	1:50	0.1% Triton
IFABP	Abcam	ab7805-500	Rabbit poly	1:100	0.1% Triton
GATA6	Santa Cruz	ssc-9055	rabbit poly	1:50	0.1% Triton
Trophectoderm:					
CDX2 (only early)	BioGenex/Novacastra	MU392-UC	Mouse, IgG	1:100	0.1% Triton
HCGbeta	Abcam	ab400-500	Mouse, IgG	1:100	0.1% Triton
Germ Cell:					
MVH (VASA)	Abcam	ab13840	Rabbit poly	1:100	0.1% Triton

Secondary Antibodies are from Molecular Probes and are conjugated to Alexa fluorophores. We use Alexa 488 (Green), Alexa 555 (orange-red), and Alexa 647 (far red). These match the best with our confocal using Multitrack settings.

Nuclear counterstains available for confocal work:

SytoxGreen (green)
SytoxOrange (orange-red)
TOPO-3 (far red) or SytoxRed
(All are from Molecular Probes.)

REMEMBER - SytoxGreen and SytoxOrange will be detected in multiple channels under epifluoresence using the filters available on our scopes. However, these can be used with confocal detection without spill-over.

REAL-TIME RT-PCR PROTOCOLS & MARKERS

Scott Noggle, doc. version 1.2 2-7-10

General experimental design: Cells are harvested from duplicate or triplicate samples. Total RNA was isolated using RNAeasy kit (QIAGEN, Cat. No. 74104). 1 ug RNA is used for cDNA synthesis with SuperScript III First-Strand system (Invitrogen, Cat. No. 18080-051) and Oligo (dT) primers and the resulting cDNA is diluted to the final volume of 200 ul. 1 ul of the cDNA dilution and 500 nM of forward and reverse primers are used for each 10 ul PCR reaction. Quantitative real-time PCR is performed using the LightCycler SYBR Green Master kit (Roche, Cat. No. 04707516001) and Mx3000p QPCR system (Stratagene).

The primer sequences for endogenous and transgene versions of the reprogramming factors are listed in the following table. These are used to check for silencing of the retroviral transgenes in iPS lines.

GENE	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'
Oct 4 (endogenous)	CCCCAGGGCCCCATTTTGGTACC	GGCACAAACTCCAGGTTTTC
Sox2 (endogenous)	ACACTGCCCCTCTCACACAT	GGGTTTTCTCCATGCTGTTTCT
Klf4 (endogenous)	ACCCACACAGGTGAGAAACCTT	GTTGGGAACTTGACCATGATTG
C-Myc (endogenous)	AGCAGAGGAGCAAAAGCTCATT	CCAAAGTCCAATTTGAGGCAGT
Oct4 (transgene)	CCCCAGGGCCCCATTTTGGTACC	AACCTACAGGTGGGGTCTTTCA
Sox2 (transgene)	ACACTGCCCCTCTCACACAT	AACCTACAGGTGGGGTCTTTCA
Klf4 (transgene)	GACCACCTCGCCTTACACAT	AACCTACAGGTGGGGTCTTTCA
C-Myc (transgene)	AGCAGAGGAGCAAAAGCTCATT	AACCTACAGGTGGGGTCTTTCA
B2M	TAGCTGTGCTCGGGCTACT	TCTCTGCTGGATGACGCG

These primer sequences are used for pluripotency markers and germ layer representation in embryoid bodies:

MARKER	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'	SIZE	CROSSREACT WITH MOUSE?
Pluripotency:				
Oct4	CAAGCTCCTGAAGCAGAAGAGGAT	CTCACTCGGTTCTCGATACTGGTT	275	F=Y R=N
Nanog	CCGGTCAAGAAACAGAAGACCAGA	CCATTGCTATTCTTCGGCCAGTTG	214	F=N R=N
Sox2 (Scott-microarr.)	TCAGGAGTTGTCAAGGCAGAGAAG	GCCGCCGCCGATGATTGTTATTAT	172	F=Y R=N

MARKER	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'	SIZE	CROSSREACT WITH MOUSE?
Ectoderm:				
Cytokeratin (Melton)	AGGAAATCATCTCAGGAGGAAGGGC	AAAGCACAGATCTTCGGGAGCTACC	782	F=N R=N
Sox1	GAGATTCATCTCAGGATTGAGATTCTA	GGCCTACTGTAATCTTTTCTCCACT	94	F=N R=N
NFH (Melton)	TGAACACAGACGCTATGCGCTCAG	CACCTTTATGTGAGTGGACACAGAG	397	F=N R=N
Mesoderm:				
Brachyury	CACCTGCAAATCCTCATCCTCAGT	TGTCATGGGATTGCAGCATGGA	188	F=N R=N
Goosecoid	CGCCTCGGCTACAACAACTACTTCTA	ACGTTCATGTAGGGCAGCATCT	193	F=N R=Y
Chordin	TGTGAGCGGGATGACTGTTCACT	AAGAGCCTTCGGCTTCTTTCTCCA	141	F=N R=N
Cardiac actin	TCTATGAGGGCTACGCTTTG	CCTGACTGGAAGGTAGATGG	668	F=Y R=N
Endoderm:				
Gata6	TTTCCGGCAGAGCAGTAAGAGG	CCGTCAGTCAAGGCCATCCA	215	F=Y R=Y
IFABP (Pedersen)	TGCCTAGAGGCTGACTCAACTGAAA	CCTTTTTAAAGATCCTTTTGGCTTC	420	F=N R=N
Sox17	GGCGCAGCAGAATCCAGA	CCACGACTTGCCCAGCAT	60	F=N R=N
FoxA2	CGTTCCGGGTCTGAACTG	ACCGCTCCCAGCATACTTT	76	F=N R=Y
CXCR4	CACCGCATCTGGAGAACCA	GCCCATTTCCTCGGTGTAGTT	78	F=N R=N
Trophectoderm:				
hCG beta	ATCACCGTCAACACCACCATCTGTG	AGAGTGCACATTGACAGCTGAG	198	F=N R=N

MARKER	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'	SIZE	CROSSREACT WITH MOUSE?
House keeping				
Beta-2-microglobulin	TTCTGGCCTGGAGGCTATC	TCAGGAAATTTGACTTTCCATTC	85	F=N R=N
TBP	GCTGGCCCATAGTGATCTTT	CTTCACACGCCAAGAAACAGT	59	F=N R=N
ATP5O	ACTCGGGTTTGACCTACAGC	GGTACTGAAGCATCGCACCT	86	F=Y R=N
UBC	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT	?	?
HPRT	TGACCTTGATTTATTTTGCATACC	CGAGCAAGACGTTCAGTCCT	101	F=N R=N

FLOW CYTOMETRY ANALYSIS OF HESCS AND IPSCS

David Kahler doc. version 2.1 3-3-10

Introduction: Characterization of cell lines by flow cytometry can yield information about the health and differentiation status (as defined by cell surface receptor or nuclear transcription factor expression) of hES / iPS cells under defined culture or experimental conditions. Moreover, this information can be obtained rapidly with minimal effort from a low cell numbers that can be spared during routine passaging. For these reasons, flow cytometry can be a valuable technique once characterization panels have been properly designed and validated. This section will discuss a method of designing flexible characterization panels to assess the undifferentiated / differentiated status of hES / iPS cells in culture.

Summary of Protocol: The key to a successful characterization assay is to first accurately define the goals of the assay. This will ensure that all the relevant markers are included and the most meaningful information can be extracted from the experiment from the minimum number of samples using the minimum amount of reagents. Defining the goals of the assay will also ensure that the appropriate experimental and staining controls are included in the characterization panel and allow for the accurate and efficient collection, analysis, and interpretation data. Preparation of antibody cocktails according to the characterization panel can be performed concurrently with the dissociation protocol or a few hours in advance. It is helpful to have a partner to assist in cell preparation so that cells are ready to be added to the antibody cocktails immediately following dissociation. Antibody cocktails are made up at twice the concentration in staining buffer so that an equal volume of staining buffer containing the dissociated cells added to the cocktail creates the correct staining concentration. Cells should be added to the tubes or wells containing the antibody cocktails quickly, ideally with a multichannel pipet in order that all samples incubate for the same time period. Allow the cells to incubate at room temperature, protected from light for 15 minutes followed by one wash in ice cold buffer. Samples should then be resuspended in 300-500µl of ice cold buffer depending on cell number and stored on ice protected from light and analyzed immediately. It is possible to fix the cells in 4% PFA for 10-20 minutes at room temperature and stored at 4°C if analysis cannot occur immediately. However, strong fixation can introduce artifacts such as autofluorescence and alter the forward and side scatter properties, and binding ability of antibodies to their epitopes.

CELL DISSOCIATION FOR FACS ANALYSIS

Dispase Recipe:

- is in powder form 1mg/ml
- so to make up 10ml take 10mg dissolve in 10ml of media your cells are growing in (mTesR1) all in a 15ml conical tube.
- Warm media in 37C water bath
- Filter to new 15ml conical in a filter syringe
- Stays good for ~ week at 4C

Cell prep for FACS analysis:

1. aspirate off media
2. add dispase (2ml) for 6 well plate or 1ml for 12 well plate
3. put in incubator – let sit for 5 minutes & check it
4. look for edges of cell to curl
5. aspirate dispase

6. add PBS (1 – 1.5ml) in 12 well or (2ml) PBS in 6 well plate.
7. Care not to wash off cells.
8. add 0.5ml for (12well) of acutase or 1ml (6well) acutase
9. pipet with 1ml (resuspend) + check to make sure no clumps
10. neutralize with an equal volume of media
11. count hemocytometer
12. move to 15ml conical bring up to 10ml w/ media.
13. Spin @ 660rpm for 8 minutes
14. Aspirate media of cell of cell pellet.
15. Resuspend in FACS buffer.

GENERAL STAINING PROTOCOL

1. Define the goals of the assay.
2. Design a characterization panel including:
    a. Unstained, Compensation, Isotype and/or FMO controls
    b. Pluripotent and Differentiation markers (surface and intracellular)
3. Prepare single cell suspensions of samples to be included in the analysis.
4. Prepare antibody cocktails at 2x concentration in 50µl.
    a. Deliver antibody cocktails to 75x12mm (FACS) tubes or 96well plates.
    b. Store protected from light.
5. Add 50µl of single cell suspension (10^5 – 10^6) to each well containing Ab cocktails and mix gently.
6. Incubate for 15-30 min in dark at (RT / +4°C / Ice)
7. Wash 1x with 1ml ice cold buffer and centrifuge at ~300g (1200 rpm) 5 min
8. Remove supernatant and resuspend cells in 300-500µl in FACS tubes
9. Place tubes on ice and keep protected from light prior to analysis
10. Analyze immediately following staining or fix w/4% PFA in PBS

Defining Goals: The key to designing a successful characterization assay is to first accurately define the goal of the assay. If the goal is to assess viability following changes in culture or experimental conditions, very few cells will be required and preparation and analysis time will be short. If the goal is to detect changes in differentiation status over time, appropriate staining controls will be required and therefore cell numbers and preparation time will be increased. If the goal is to sort viable cells for culture, several pilot characterization experiments may be required to accurately identify populations of interest. Other factors to consider are the availability of the antibodies for your application and if they can be conjugated to fluorochromes that can be detected by the instrument. If you do not own your own flow cytometer or cell sorter, it is important to consult with an

operator in your core lab to determine which fluorochromes can be detected by the instrument. Also, there may be restrictions on the types of cells that can be run unfixed on the instruments.

Designing a Characterization Panel: Control samples are an important component of every experimental procedure and ensure that instrumentation is working properly and that experimental conditions are such that biologically and statistically significant differences can be detected. The design of the characterization panel should include control samples which allow for the setup and calibration of the flow cytometer according to the fluorochromes used in the assay, and for the accurate placement of gates which discriminate negative from positive events. Compensation controls consist of unstained cells and cells which are stained for each color to be detected by the instrument. Unstained cells are used to set the initial detector voltages, and tubes containing cells which are stained for each color to be detected are required to set compensation values if the emission spectra of the fluorochromes overlap. The online spectral fluorescence viewers listed in the materials section are helpful in determining if the fluorescence combinations in the characterization panel can be compensated from each other, thus allowing accurate data analysis. Staining controls consist of two types – fluorescence minus one (FMO) and isotype controls. Fluorescence minus one (FMO) controls are tubes of cells stained with all the markers of interest minus one marker. FMO controls are compared to fully stained tubes to determine the gating criteria which discriminates the negative from the positive events. Isotype controls are included to determine the extent of non-specific antibody binding that occurs by cells. Isotype controls consist of cells stained using an antibody for an irrelevant antigen but consisting of the same isotype as the specific antigen of interested. In the flow cytometry community, much debate occurs over which control is the most appropriate. Inclusion of both controls is useful in initial characterization panels to rule out artifacts caused by autofluorescence and nonspecific antibody binding. Experimental controls are also an important consideration in designing stem cell characterization panels particularly if cells will express EGFP following treatments.

There are three overall steps involved in characterizing stem cell lines by flow cytometry:

1. Preparation of single cell suspensions from cultured cells.
2. Preparation of antibody cocktails and staining the cells.
3. Acquisition and analysis of the stained samples.

Preparation of single cell suspensions from cultured cells: Because individual cell lines grown on feeder layers or growth matrices respond differently to various dissociation protocols, it is important that the optimal conditions for the preparation of single cell suspensions from individual stem cell lines are already in place prior to the development of the characterization panel. It is important to preserve both viability and cell surface receptor expression particularly in cases of cell sorting for re-culture. Therefore, several dissociation techniques should be tested (Accutase®, Trypsin, TrypLE®) to identify the most effective protocol that produces a single cell solution which retains the highest level of cell surface receptor expression. In the case of multicolor characterization panels which include intracellular staining for transcription factor expression, it is important to verify that the fixation and permeabolization steps required to detect intracellular / nuclear components do not destroy the epitope of the surface receptors of interest.

Preparation of antibody cocktails and staining the cells: Antibody cocktails should be prepared just prior to staining the cells, although they may be prepared in advance and stored for 2-3 days without degradation of signal. This can be advantageous for time course experiments where cells will be treated with the same panel of antibodies over the course of a few days. Preparation of a sufficient volume of the same cocktail would reduce the variation in staining intensity introduced by preparing different cocktails each day of analysis. Prior to mixing, antibodies should be spun down in their tubes in order to prevent the production of “false positive” results or other fluorescent artifacts caused by highly fluorescent protein aggregates. Antibody cocktails should be prepared in a sterile environment to prevent contamination of the source antibodies. This is particularly important when preparing cocktails the will be used to sorting cells for subsequent culture and experimentation. Antibodies should also be kept on ice and protected from light to prevent degradation of fluorescence intensity, an important consideration especially if tandem dyes such as PE-Cy5, PE-Cy7 or PerCP-Cy5.5 are being used in the panel. Staining protocols consist of incubating single cell suspensions under conditions shown below in the table of staining parameters. Manufacturers suggested protocols usually are based on staining 10^6 cells with 5-20µl of each antibody in 100µl of staining buffer on ice protected from light for 30 minutes. The cell number and antibody volume is usually excessive for stem cell characterization and it is important to titrate individual antibodies down to the minimum volume that produces the maximum

mean fluorescence intensity (MFI) at a given number of cells. Most directly conjugated antibodies work well when used at 1-2µl per 10^5 cells in 100 µl total staining volume.

Data Acquisition and Analysis: As discussed in the previous section, individual cell lines cultured under different conditions will respond differently to various dissociation protocols. It is important that cells remain as a single cell solution during the staining period and not clump together, thus restricting the ability of the antibodies to bind their epitopes and producing inaccurate staining profiles. Clumping is detrimental to the flow cytometer as clumps of cell plug the sample tubing and flow cell and are difficult to remove and require extensive flushing of the instrument. To prevent clumping, it may be necessary to increase the concentration of serum in the buffer from 1-5% v/v and keep the cells on ice once dissociated. Filtering the cell suspension through cell strainers or filter cap FACS tubes shown in the suggested labware table just prior to placing samples on the cytometer greatly reduces the chance of plugging the machine.

Example of a 3 Color iPS Cell Characterization Panel:

Cell Type: 1018M iPS cell line transformed with 4 factors and cultured for 2 months on MEFs

Goal: Determine the viability and pluripotent status of the 1018M iPS cell line. We are interested in assessing which cells are negative for CD13 and double positive for SSEA4 and SSEA3 or SSEA4 and Tra-1-60.

Staining Layouts: Because the emission spectra of FITC and PE overlap, they will be required to be compensated. AlexaFluor 647 (APC) does not overlap with FITC or PE but will be included as a compensation control along with the unstained sample in order to use the automatic compensation feature of the DIVA® software.

Compensation Controls:

Tube #	Marker_1	Color	BD Cat #	Volume
1	CD13	PE	555394	1
2	SSEA4	AF 647	560219	1
3	SSEA3	FITC	560236	1
4	Tra-1-60	AF 488	560173	1

Characterization Panel:

Tube #	Marker_1	Marker_2	Marker_3	Color	BD Cat #	Volume	Ab Vol
0	Unstained					200	
1	CD13			PE	555394	1	4
5	"	SSEA4		AF 647	560219	1	3
6	"	"	SSEA3	FITC	560236	1	1
7	"	"	Tra-1-60	AF 488	560173	1	1

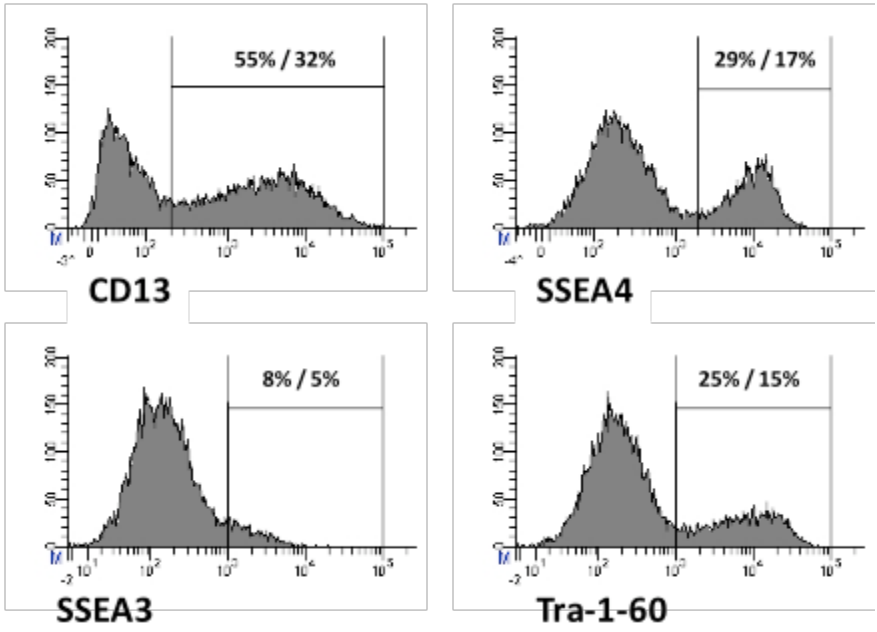
Table of Common Staining Parameters

Cell Number	$5*10^4$ - 10^6
Staining Volume	50 – 100 µl
Antibody Volume	1-5 µl
Temperature	4 -37°C
Incubation Time	15 – 60 minutes
Serum Concentration	0.5 – 5%

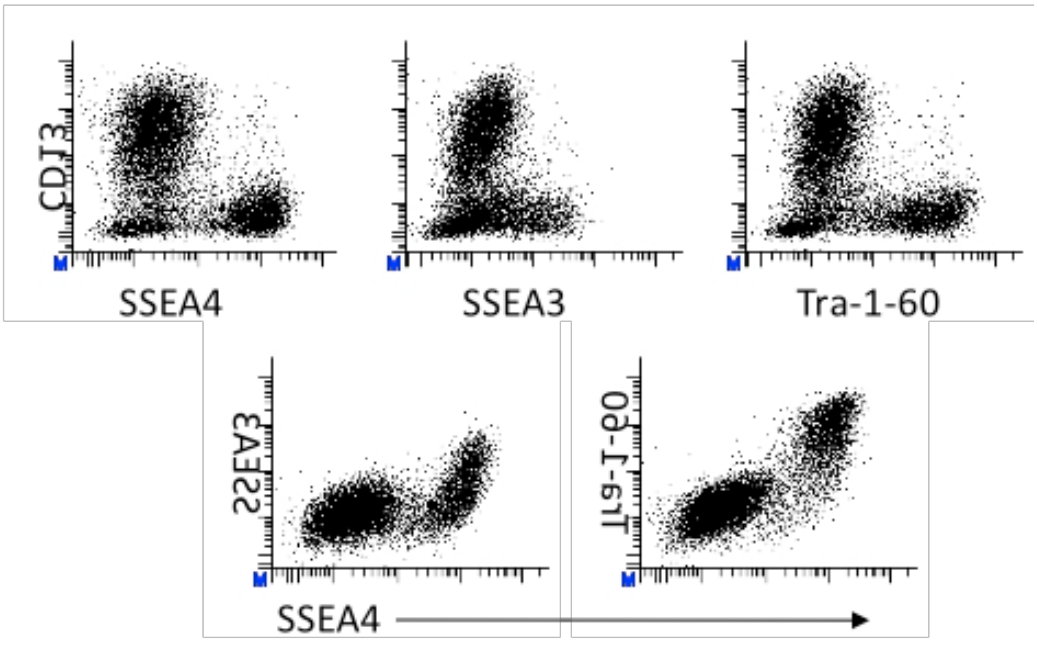
RESULTS OF IPS CELL CHARACTERIZATION BY FLOW CYTOMETRY

Single Parameter Analysis of Cell Surface Marker Expression Using Histograms

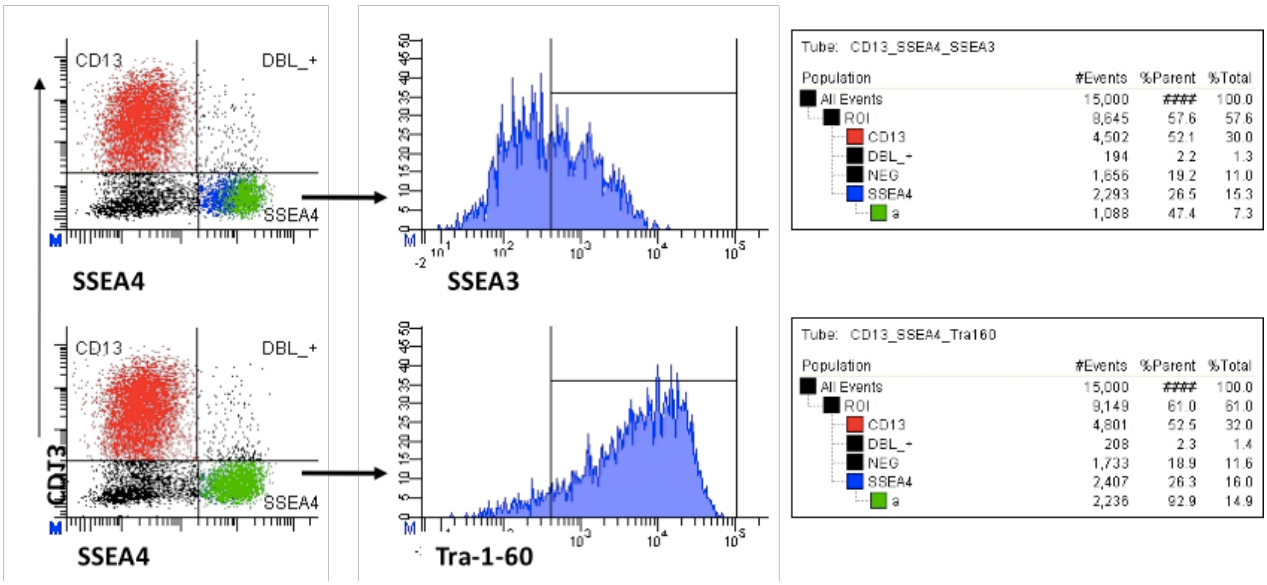
(% of ROI Gated Cells / % of Total Cells Analyzed)



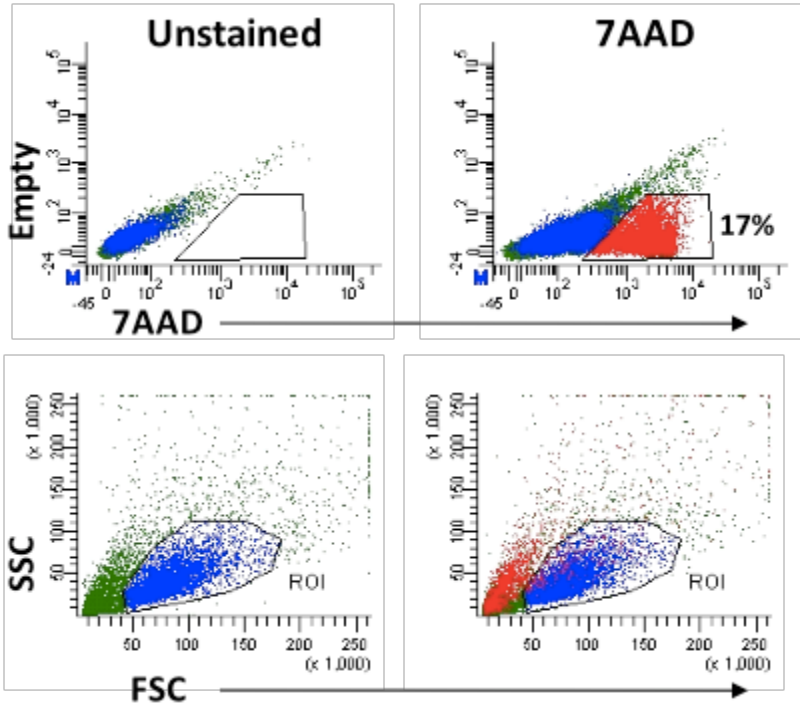
Two Parameter Analysis of Cell Surface Marker Expression Using Dotplots



Three Parameter Analysis of Cell Surface Marker Expression Using Dotplots and Histograms



Viabilty Assay Using 7AAD



MATERIALS

Staining Buffer

General staining buffer for dissociation, analysis and sorting of neural stem cells
Prepare in sterile environment, vacuum filter through 0.22 µm and store at +4°C

Components of Staining Buffer

Reagent	Manufacturer	Catalog #	Volume	Final Concentration
DPBS	Invitrogen	14190250	460.0 ml	500 ml
BSA Fraction V Solution (7.5%)	Invitrogen	15260037	33.0 ml	0.5%
Penicillin-streptomycin, liquid	Invitrogen	15070063	5.0 ml	100 U/ml
0.5M EDTA, pH 8.0	Invitrogen	15575038	2.0 ml	2mM
Glucose	Sigma	G6152	1.8 g	20mM

List of Suggested Labware

Item Description	Manufacturer	Catalog #	Fisher #
Polystyrene 12x75 5ml FACS Tubes	BD	352052	149596
Polystyrene 12x75 w/snap cap	BD	352058	149591A
Polypropylene 12x75 w/snap cap	BD	352063	1495911A
Filter cap FACS tubes	BD	352235	0877123
U-Btm 96 well plates non-TC treated	BD	35117	877254
40µm BD Falcon Cell Strainers	BD	352340	087711
Falcon 15ml conical tubes	BD	352097	1495970C
Unwire Rack 13mm tubes	Nalgene	59760313	14809131
Falcon 50ml conical tubes	BD	352098	1495949A
Disposable Sterile pipets individually wrapped	Fisher		1371120

Online Resources

Introduction to Flow Cytometry Training Video

http://www.bdbiosciences.com/immunocytometry_systems/support/training/online/

Flow Cytometry Tutorials / Presentations and spectral viewers

http://www.cyto.purdue.edu/

http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html

http://www.bdbiosciences.com/colors/fluorescence_spectrum_viewer//