Khademhosseini Lab

Protocol Manual

Last updated August 31, 2006

General Lab Maintenance Protocols

-80 Freezer Maintenance

Cleaning the Filter (~3-6 months)

Open panel on the bottom front of freezer
Remove the filter
Remove dirt/debris by beating (outside)
Rinse filter with water
Replace

Cleaning Ice from Inside Doors (~weekly)

Scrape ice off of inside doors, focusing on the area where the outside door seals (build up of ice prevents door from sealing)

- eppendorf tube holder is effective for scraping off doors
- lab marker is effective for scraping off seal area

Isolation of RNA from Cells or Constructs

1. Rinse cells/constructs with PBS (3x, 5 min ea.)

CONSTRUCTS

2. Transfer constructs to 2 ml eppendorf tube
3. Add 0.5 ml TRI reagent and homogenize
4. Add 0.5 ml TRI reagent to tubes to total 1 ml added
5. Vortex for 15 s. and react 10 min.

CELLS

2. Add 1 ml TRI reagent to cells and react for 5 min
3. Transfer TRI reagent to 2 ml eppendorf tube
4. Vortex for 15 s. and react for 10 min.

ALL

6. Add 0.2 ml chloroform to tubes, vortex 15 s. and react for 10 min
7. Centrifuge at 12000g for 15 min. at 4 ºC
8. Transfer aqueous phase (top) to new eppendorf tube and add 0.5 ml isopropanol
9. Vortex 15 s. and react at RT for 10 min.
10. Centrifuge at 12000g for 10 min. at 4 ºC
11. Remove supernant (carefully) and wash pellet with 1 ml 75% ethanol in 0.1% DEPC water
12. Vortex sample for 15 s. and centrifuge at 7500g for 5 min. at 4 ºC
13. Store samples in 75% ethanol at 4 ºC (up to 1 week)

Fluorescent Microscope Operation

Power: Turn on mercury lamp power first. Then turn on the rest (halogen light, computer, general power supply) in any order. We can leave the computer on always if desired. Turn off mercury lamp last when shutting down.

Phases: Ph1 → 10X and 20X objectives
Ph2 → 40X objective

Ports: 5 → digital camera/computer
1 → eyepiece

Eyepiece set: O → observation
C → closed
M → magnifies image to your eyes by 2.5X
B → sets focus on back of the lens

Filters: UV → blue emission
B → blue excitation/ green emission
G → green excitation/ red emission
(lower wavelength excitation > higher wavelength emission)

Condenser Adjustment: Adjusts image resolution and location
z-plane → use large black knobs on the headpiece
x,y-planes → use small silver knobs below the big black knobs
radius of image → use field diaphragm lever (on right, above black knobs)

Magnification: Knob on lower right side of scope magnifies image to the camera as well as the eyepiece. It goes from 1X to 1.5X. Decreases image quality when using fluorescence.

Fluorescence: Turn off halogen lamp (switch on lower left of scope). Open switch (on right side next to filters) which goes from "O" to "C". This lets the fluorescent light through.

Focal Plane: The ring around the top of each objective adjusts the focal plane and increases image resolution. Purpose: adjust for the thickness of the platform your sample rests on. How to use: Adjust ring slightly, then refocus, then adjust ring again. Repeat until image resolution is maximized.

Fluorescent Microscope Image Acquisition

Startup Program: Open *Spot Advanced* program on desktop

Live image viewing: Click on *Live* button on far right of main screen. *Controls* button allows adjustments of *gamma* (grayscale range), *contrast* and *brightness* (essentially affecting exposure time). *Snap* button captures the current image.

New exposure setup: Setup→ Image Setups → Add → (any)
Change exposure time to manual.
Then click on manual on left side, and set an exposure time.
Most of the other settings remain the same for all.

Color: Edit → Set Palette → (Choose palette to match stain)
Setup → Palettes… (Allows creation of new palette)

Adjust Contrast: Edit → Adjust RGB → Adjust Histogram (this is the best option)
Edit → Adjust RGB → Contrast (also may be helpful)
Edit → Adjust RGB → Adjust Gamma (adjusts grayscale range)

Multiple Stains: Create separate images using the different filters.
Then merge the images: Edit → Merge Images
Can also merge light images with fluorescent images

Sharpen Image: Edit → Filter → Unsharp image
Be careful, only so much is reasonable to believe.

Add Scale Bar: Edit → Set Calibration

Plasma Cleaning

This procedure is used to clean surfaces and enhance attachment of PDMS to glass or PDMS to PDMS.

1. Clean all substrate surfaces with Scotch tape.
2. Place PDMS mold (feature side facing up) on a microscope slide. Transfer PDMS on slide into the plasma cleaner.
3. Put another microscope slide (to be attached to the feature face of PDMS mold) in the plasma cleaner.
4. Close the door to plasma cleaner, and close the valve (turn knob inward) on door to isolate inside from outside air. Set power level to medium.
5. Create vacuum by turning on pump. Watch needle on vacuum gage moving counterclockwise past the 200 mark.
6. Turn on power, slowly open valve (turn knob outward) until a purplish glow is observed.
7. Let the purplish glow persist for 25 seconds, then turn power off.
8. Open valve completely to let air in. Close valve to reestablish vacuum. Turn off pump when the needle on vacuum gage returns to its position around the 200 mark (this step is important in removing toxic gas generated in the plasma cleaner).
9. Open valve to let air in. Open door when atmospheric pressure is reached.
10. Remove PDMS mold on microscope slide and the other microscope slide.
11. Attach the empty microscope slide (the side facing up when placed in plasma cleaner) to the PDMS mold (feature, the side facing up) and squeeze for 30 seconds.
12. Remove the microscope slide lying underneath the PDMS mold (contacting the non-feature side). The PDMS mold should now be only attached to the originally empty microscope slide, with feature facing down to the slide.

Keeping a Laboratory Notebook

Purpose: To outline the key features of a lab notebook that will aid in organizing and recording experimental data. The lab notebook should also serve as a tool for planning experiments and developing experimental methods. A good lab notebook can save a lot of time!

Procedure:

1. Obtain a lab notebook number and record the number on the front of your lab notebook. Your lab notebook should be bound and have numbered pages.
2. The first 4-5 pages should be left for a table of contents. The table of contents should list the following information for each experiment: number (e.g. 1, 2, etc), date started, descriptive title, and page numbers.
3. Every entry in the notebook should contain the date, the experiment number, and a title at the top of the page. If you add comments to an entry page on a different date record the new date before commenting.

Experimental Entries:

What to record—what, when, where, why, how, and most importantly, what does it suggest?

1. Enter experiment into table of contents.
2. Purpose: The first time you do a new type of experiment you will need to describe what you plan to do, how you are going to do it, and why. Give relevant information such as the name of the technique employed and any molecules, cell type, etc involved. Subsequent experiments of the same type should note any differences in experimental technique, molecules, cells, etc. and what you hope to learn from the new experiment. You should be able to read the purpose years later and identify quickly how a particular experiment differs from other experiments of the same general kind.
3. Materials: Mostly for chemicals and any non-standard disposable lab supplies used in the experiment. The first time you perform a new type of experiment, make a table with the following information: name, supplier, catalog number, and lot number. Note any chemicals that are stored at temperatures other than room temperature or any special chemical or material handling requirements. Subsequent experiments can reference the page number where the materials are listed, but should note any new materials or new lot numbers used. Keep in mind that materials purchased from suppliers can vary from lot to lot. It may be easier to troubleshoot why something did not work if you have a good record of all variables.
4. Procedure: List the steps involved giving specific details concerning experimental conditions. Note any observations on the effects of a particular step and what the observations might suggest. For established procedures, reference a lab protocol or lab notebook page number and only note any differences in procedure or observed

responses. Note the location of any computer data files and the file naming conventions. Fill out an information file and save the information file to the same location. Other forms of data should either be recorded directly into the notebook, glued/taped into the notebook, or have a referenced location such as a labeled paper file, videotapes, CD's, etc. What data should be recorded will vary widely with the type of experiment so you should come up with a standard way of recording important parameters. A standard table/file format will make comparisons between similar experiments easier. The first time you perform a new type of experiment note the manufacturer and model number of any equipment used. The lab manual contains specific information about each piece of equipment in the lab, so if you know what equipment you used you should be able to reference the manual if further information is required. Include any data analysis such as graphs or calculations at the end of this section. If there is a time lag between data analysis and the start of the next experiment leave a few pages to insert the analysis before recording the next experiment or note the page number where the analysis is inserted.

5. Conclusions: What did you learn from this experiment? Were the results what you expected? Why or why not? Do the results make sense? Can the experimental method/approach be improved or simplified? If so, how? What do you infer from the data and why? What are the possible explanations of an observation or result? Which possibilities are most likely? What are you assuming is happening and how will that change your experimental approach? What would you recommend for future experimental directions? Keeping track of what you think is happening and what assumptions you make can help if you later realize that something else may be happening or offers a better explanation. Always keep in mind what you are trying to learn/achieve from the experiment and whether or not the experimental results provide you with the information you want. How can you modify your approach to get what you want?

Cell and Tissue Culture Protocols

Media

NIH3T3 Media

Materials

DMEM (Red Media)
Calf Serum
Pen-Strep
Fungizone

Media Prep

1. Obtain a bottle of DMEM from cold room, place in warm water bath
2. Obtain Calf Serum, Pen-Step and Fungizone from freezer (4° C), place in warm water bath
3. Add 50mL calf serum, 5mL Pen-Strep and 1mL Fungizone to DMEM
4. Place media in refrigerator for storage.

MEF Media

Filter using a 0.2micrometers filter bottle (500mL) :

- 450 mL of Dulbecco's Modified Eagle Medium
- 50mL of Fœtal Bovine Serum (FBS)

ES Media

Pour into a filter (500mL bollte with 0.2 micrometers filter unit):

- 400mL of DMEM knockout Medium
- 5mL of Non essential Amino Acid Solution
- 1mL of 2-mercapto ethanol
- 2.5mL of L-glutamine
- 0.250mL of BFGF from a stock of 10μg/mL
- 0.5mL of LIF (10^6units)
- 100 mL of Knockout (KO)

Preparing bFGF:

- Aliquit BSA into 1 mL PS + 0.1% BSA
- Add 10 ug of bFGF to 1 mL of BSA and aliquot into 250 ul samples.

EB Medium

Pour into a filter (500mL bollte with 0.2 micrometers filter unit):

- 400mL of DMEM knockout Medium
- 5mL of non essential Amino Acid Solution
- 1mL of 2-mercapto ethanol
- 2.5mL of L-glutamine
- 100 mL of Knockout (KO)

Beta-cell (Islet) Media

M199 (500ml)
10% FBS (55 ml)
Fungizone (1ml/555ml media)

AML12 Media

225mL DMEM
225mL Ham's F12 medium
50mL FBS
5mL Pen-Strep

Feeding Cells

General Procedure

Preparation:

Place appropriate cell media in warm water bath to warm

1. In a clean sterile hood, aspirate off old media
2. Replace with new fresh media

Gelatin

1) Take the empty bottle of gelatin
2) Wash it 3 times with sterile water
3) Poor into the bottle a whole new bottle of sterile water
4) Add 0.5g of gelatin
5) Wrap the cap of the bottle with aluminum foil and with the autoclave tap
6) Autoclave the bottle for 45 min

Passaging cells

General Procedure

Materials:
- cell culture media
  VICs: M199 + 75 ml FBS + 5 ml Pen-Strep + 1 ml gentamicin + 5 ml L-glutamine
  3T3s: DMEM + 50 ml FBS or Calf Serum + 5 ml Pen-Strep
- phosphate buffered saline (PBS)
- trypsin/EDTA

Preparation:
- warm media, PBS, and trypsin to 37°C in water bath
- wipe laminar flow hood down with 70% ethanol
- wipe all containers that enter hood with 70% ethanol

Procedure: (for 10 mm dishes)
1. Aspirate old media from dish.
2. Add 8 ml PBS to dish. Always tip dish to the side when adding solutions so as not to shear the cells attached to the bottom surface.
3. Aspirate off PBS.
4. Add 5 ml of trypsin. Tilt dish to ensure that the entire bottom surface is covered with solution.
5. Place in 37°C incubator for 2-6 minutes.
6. Remove dish from incubator and gently tap it to loosen the cells. Inspect dish either macroscopically or microscopically to ensure that all cells have been cleaved from the surface. If not, either tap dish a few more times or return to incubator for 1-2 minutes longer.
7. Add 4 ml of media to the dish.
8. Remove all contents of the flask and place in 15 ml centrifuge tube.
9. Rinse dish surface with 4 ml PBS or 4 ml media and add to same centrifuge tube.
10. Tightly cap centrifuge tube, make appropriate counterweight, and centrifuge at 2000 rpm for 5 minutes (3T3s) or 1000 rpm for 6 minutes (VICs).
11. While the cells are centrifuging, prepare new dishes for cells to be passaged into. Add 8-10 ml media to each dish and label appropriately.
12. There will be a visible cell pellet at the bottom of the tube following centrifugation. Aspirate off as much supernatant as possible without disturbing the cells.
13. Resuspend cells in media (normally 5-6 ml). If seeding cells for an experiment, remove 10 μl and perform a hemacytometer count or coulter count.
14. Add the appropriate amount of cell suspension to each dish (1:3 is the recommended passage ratio for VICs; 1:50 is good for 3T3s) and place dishes in the incubator.

Clean-up
- All materials that have touched cells or media must be thrown in biohazard waste.

- Materials that have only touched trypsin, buffer, etc., can be thrown in regular trash.
- Wipe down hood with 70% ethanol.
- Turn on UV light, turn off blower, and close sash.

MEF cells

I. Seeding MEF cells

1) Take the cells from the minus 80
2) Leave them in the bath until you see a small ice ball; don't leave them completely melt
3) Slowly take the cells with the Pasteur Pipette
4) Put the cells in a 15 mL Falcon tube
5) Add 5mL of MEF Medium drop by drop
6) Spin the tube at 1000tr/min for 5 minutes
7) Take a flask, add 15mL of MEF medium, write the date and P0 (passage0)
8) Take the tube and remove the medium and resuspend the cells with 2mL of medium
9) Seed the cells in the flask and leave them in the incubator.

Preparing mitomycin:
- 250 mL of DMEM
- 2mg of mitomycin

II. Passage MEF with mitomycin

1) Remove the media that is in the flask
2) Add the entire tube of motomycin (about 6 to 7 mL)
3) Leave the flask in the incubator for 2 hours
4) After 2 hours, remove all the mitomycin
5) Wash 4 times with PBS
6) Remove the last PBS washing
7) Add 2mL of trypsin
8) Put the flask in the incubator for about 2 minutes
9) Take the flask from the incubator and add 5 to 6 mL of MEF Medio to stop the trypsin
10) Pipette up & down around 10 times and and put in a 15 mL falcon tube
11) Spin down at 1400 for 5 minutes
12) During that time, take plates from the incubator, remove the gelatin and add MEF Medio
13) Resuspend the cells and put them in the plates : usually 1 flask into 4 10cm plates

Passage ES with collaganase

1) Take out the media from the plates
2) Add 4mL of collagenase in each plate
3) Leave the flask in the incubator for 30 to 45 minutes
4) Add 5 mL of ES media
5) Wash the plategently to remove the ES colonies from the plate
6) Put them in a 15mL falcon tube
7) Wash a second time with ES media
8) Spin down at 700 for about 3 minutes
9) During that time, take new plates from the incubator, remove the gelatin and add 10mL of ES Medio
10) Resuspend the pellet by pipet up & down strongly
11) Spin down at 700 for about 3 minutes
12) Up& down strongly
13) Resuspend the cells and add to the plates
14) Put the plates in the incubator

For EB's, put EB medium in a petry dish

Use Colleganse type 4 from Gibco – Invitrogen
1 mg collegenase /mL of DMEM without serum and then filtuer with 0.2 um filter and only use for 2 weeks.

Passage ES with trypsin

1) Take out the media from the plates
2) Wash one time with PBS
3) Add 3mL of trysin (0.25 or 0.1)
4) Leave the flask in the incubator 5 minutes
5) Add 6 mL of TNS
6) Up & down many times to remove all the cells
7) Put them in a 15mL falcon tube
8) Pipet **strongly**
9) Take a Pasteur pipet and remove the gelatin
10) Spin down at 700 for about 3 minutes
11) During that time, take new plates from the incubator, remove the gelatin and add 10mL of ES Medio
12) Resuspend the cells and add to the plates (usually split 1 to 4)
13) Put the plates in the incubator

For EB's, put in EB medium in petry dish

Freezing Cells

Materials:

- Cell culture media
- PBS
- Trypsin
- FBS
- Sterile-filtered DMSO
- Cyrovials
- Freezing media – 45% media + 50% FBS + 5% DMSO

Preparation:

- Warm media, trypsin, PBS, and FBS in water bath
- Wipe laminar flow hood down with 70% ethanol
- Wipe all containers that go into hood with 70% ethanol

Procedure:

1. Aspirate old media from dishes
2. Add 8 ml PBS
3. Aspirate off PBS
4. Add 4 ml trypsin
5. Place dish in incubator for 2-6 minutes
6. Remove dish from incubator and gently tap to loosen cells. Make sure all cells have lifted off dish – if not put back in incubator for 1-2 more minutes.
7. Add 4 ml media to dish
8. Remove all liquid of dish to 15 ml centrifuge tube
9. Rinse dish with PBS and move to centrifuge tube
10. Centrifuge at 1000 rpm for 5 min.
11. Aspirate off as much supernatant as possible.
12. Resuspend cells in 1 ml media.
13. Perform cell count (Coulter counter) to determine number of cryovials needed (1-2 million cells/cryovial)
14. Add appropriate amount of media and FBS to cell suspension. Add the DMSO last as it is toxic to cells.
15. Add 1 ml of cell suspension to each cryovial.
16. Place cryovials in -80 ºC freezer

Thawing Cells

1. Remove cells from -80 freezer
2. Thaw in warm water bath until a small chunk of ice is left in the vial
3. Add small amount of media to vial so as to dilute DMSO
4. Move vial contents to 15 ml centrifuge tube and further add media until reaching 10 ml total
5. Centrifuge at 1000 rpm for 2 min
6. Aspirate
7. Resuspend in proper media and incubate

Use of Hemacytometer to count cells:

1) Dilute cells 1:1 with trypan blue solution
    a. Use separate eppendorf tube
    b. 100 µL cells, 100 µL trypan blue is a good volume
2) Mix well and allow dye to permeate dead cells (~ 5 min)
3) Place cover on hemacytometer
4) Pipette cell/dye mixture into side channel until one side under cover is filled
5) Count only living cells (not blue) in large central block
6) Multiply by 2 to account for dilution
7) Multiply by 10^4 to obtain cells/mL
8) Repeat with other side of hemacytometer for average
9) Clean hemacytometer and cover by wiping with ethanol on a Kimwipe

Notes:

1) Total # cells/mL of original cell suspension = (# of cells in 4 boxes/4)x10,000xdf
2) Cell viability = # alive cells/ (# alive cells + # dead cells)

Tethering cells to glass coverslip

1) Immerse coverslip with fibronectin solution (20-200 ng/mL concentration)
2) Incubate for at least 15 min
3) Remove old media from cells
4) Add enough trypsin (1X-10X concentration) to cover bottom of flask
5) Wait a few minutes until cells no longer adhere to flask (optional to place in incubator or to tap flask gently)
6) Dilute with PBS and pellet cells
7) Remove supernatant and resuspend cell pellet in desired amount of fresh media
8) Remove fibronectin solution from coverslips
9) Tether cells to coverslip by incubating cells with fibronectin coated coverslips for several hours (~2 hours at least)

Care of HL-Cardiomyocytes

Claycomb Lab

Please ignore the "Preparation," "Substrate Coating," "Freezing" and "Thawing" instructions included in the Product Information accompanying the Claycomb Medium (JRH Biosciences, Cat # 51800). Our instructions are as follows:

Supplemented Claycomb Medium	ml	Final Concentration
Claycomb Medium	87	
Fetal bovine serum	10	10%
Penicillin/Streptomycin	1	100 U/ml:100 μg/ml
Norepinephrine (10 mM stock)	1	0.1 mM
L-Glutamine (200 mM stock)	1	2 mM

- ♥ Wrap the Claycomb Medium bottle in aluminum foil, since the medium is extremely light sensitive.
- ♥ Supplemented Claycomb medium is good for two weeks, at which time L-glutamine is replenished.

Norepinephrine [(±)-arterenol], mw 319.3

- ♥ Norepinephrine is made up in 30 mM ascorbic acid.
- ♥ Make up 100 ml of 30 mM ascorbic acid by adding 0.59 g ascorbic acid to 100 ml of cell culture grade distilled water.
- ♥ Add 80 mg norepinephrine to 25 ml of the 30 mM ascorbic acid.
- ♥ Filter sterilize using a 0.2 μm Acrodisc syringe filter.
- ♥ Aliquot in 1 ml volumes into sterile microtubes with screw caps, and store at -20°C. This is 10 mM (stock) norepinephrine. Use 1 ml of stock per 100 ml medium for a 0.1 mM final concentration.
- ♥ Norepinephrine needs to be made up fresh monthly.

L-Glutamine

L-Glutamine comes as a 100× solution, and is aliquoted into working volumes and frozen.

Freezing Medium

- ♥ Freezing medium is made up of 95% FBS/5% DMSO.
- ♥ This can be stored up to a week at 4°C.

Soybean Trypsin Inhibitor

- ♥ Weigh out 25 mg of soybean trypsin inhibitor, and place into a beaker containing 100 ml of Dulbecco's phosphate buffered saline (PBS) until dissolved.
- ♥ Filter sterilize (using a 0.2 μm syringe filter) into a 100 ml bottle.
- ♥ This is good for a month at 4°C.

Pre-coating flasks

Gelatin/Fibronectin

- Weigh out 0.1 g gelatin and place into a 500 ml glass bottle.
- Add distilled water to the 500 ml mark, and autoclave. This gelatin will go into solution while being autoclaved. The concentration of gelatin in 0.02%.
- Fibronectin is received in a tube as 5 ml liquid. Dilute 1 ml fibronectin in 199 ml of 0.02% gelatin. Mix gently, and immediately aliquot 6 ml per 15 ml centrifuge tube. Freeze aliquots at -20°C.
- Before culturing cells, coat tissue culture flasks with gelatin/fibronectin (2 ml/T25 or 6 ml/T75 flask). Cap the flasks, and incubate at 37°C overnight.
- Remove the gelatin/fibronectin by aspiration the next day just before adding cells to the flasks.

Culturing cells

- Cultures are fed (5 ml/T25 flask) with supplemented Claycomb Medium every weekday.
- To avoid feeding the cells on weekends, 10 ml of supplemented Claycomb Medium is added to each T25 flask on Friday afternoons; this medium is not changed until the following Monday morning.

Passaging: this is the procedure for a 1:2 split.

After the cells first arrive, it is recommended that they be split when they reach confluency.

- Split one of the T25 flasks 1:2, resulting in two T25 flasks. This set of two T25 flasks will be your "working" set of cells.
- **Split the other T25 flask 1:3, and place the contents into one T75 flask** (protocol follows)**. After the cells in this T75 flask are confluent, they should be split into two T75 flasks. When the cells in these two flasks reach confluency, they can either be frozen** (protocol follows under "Freezing"), **or further split 1:2, resulting in 4 T75 flasks to be frozen. We recommend freezing away four flasks.**
- **Thus, within a week of receiving the HL-1 cells, you should have four cryovials of these cells frozen away for future use.**

- It is recommended that cultures be split only after full confluence.
- Rinse each T25 flask briefly with 3 ml of 0.05% trypsin/EDTA warmed to 37^{o}C (use 6 ml for T75) by pipetting the trypsin/EDTA onto the bottom of the flask (side opposite the cap), trying not to hit cells directly with the enzyme. Rinse gently and remove by aspiration.
- Add another 1.3 ml trypsin/EDTA per T25 flask (3 ml per T75). Incubate at 37^{o}C for 2 minutes.
- Remove and add fresh trypsin/EDTA. Incubate for an additional 2-3 minutes. Look at the cells microscopically, as this may only require 2 minutes.
- Examine microscopically and, if cells are still adhered, rap the flask to dislodge remaining cells.

- To inactivate the enzyme, add an equal amount (1.3 ml) of soybean trypsin inhibitor directly onto cells.
- Transfer cells from the flask into a 15 ml centrifuge tube.
- Rinse the empty flask with 5 ml wash medium (Claycomb Medium containing only 5% FBS and penicillin/streptomycin), and add to the cells already in the 15 ml centrifuge tube.
- Centrifuge at 500×g for 5 minutes.
- Meanwhile, remove the gelatin/fibronectin solution from each T25 flask, and add 4 ml supplemented Claycomb Medium/flask. Set aside.
- Remove the tube containing the HL-cardiomyocytes from the centrifuge. Remove the supernatant by aspiration, and gently resuspend the pellet in 3 ml of supplemented Claycomb Medium.
- Transfer 1 ml into each of three labeled, gelatin/fibronectin-coated T25 flask. Each flask now contains 5 ml.
- If the cells are passaged on a Friday, use 2× the volume of supplemented Claycomb Medium per flask.

Freezing:

IT IS RECOMMENDED THAT YOU FREEZE 4 OR MORE VIALS AS SOON AS POSSIBLE AFTER RECEIPT OF THE CELLS (please see note under "Passaging").
This allows you to return to this passage, and also protects you in case of contamination.

- ♥ We generally freeze the contents of one confluent T75 flask into one cryovial. (When cells are needed, this cryovial is thawed into one T75 flask.)
- ♥ Briefly rinse the T75 flask containing the HL-1 culture with 5 ml of 0.05% trypsin/EDTA warmed to 37°C. Remove by aspiration.
- ♥ Transfer 3 ml of trypsin/EDTA into the flask.
- ♥ Incubate the flask at 37°C for 2 minutes.
- ♥ Remove the trypsin/EDTA, and replace with 3 ml of fresh trypsin/EDTA.
- ♥ Incubate at 37°C for 2-3 minutes.
- ♥ Check under a microscope that cells are dislodged. If not, rap to dislodge any adherent cells.
- ♥ Add 3 ml of soybean trypsin inhibitor to the flask, and transfer the 6 ml into a 15 ml centrifuge tube.
- ♥ Rinse each empty flask with 8 ml wash medium, and add to the cells already in the 15 ml centrifuge tube. Total volume is now 14 ml.
- ♥ Centrifuge tube for 5 minutes at 500×g.
- ♥ Remove wash medium by aspiration.
- ♥ Gently resuspend each pellet in 1.5 ml of freezing medium (95% FBS/5% DMSO).
- ♥ Pipette resuspended cells into a cryovial. Place the cryovial containing the cells into a Nalgene freezing jar containing room temperature isopropanol.
- ♥ Immediately place the freezing jar into a –80° C freezer, and freeze cells at a rate of -1°C/minute.
- ♥ Six to twelve hours later, transfer the vial to a liquid nitrogen dewar.

Thawing:

- ♥ Gelatin/fibronectin-coat a tissue culture flask overnight in a 37°C incubator.
- ♥ Next morning, remove the gelatin/fibronectin from the culture flask, and replace with 10 ml of supplemented Claycomb Medium. Place this flask back into incubator.
- ♥ Transfer 10 ml wash medium into an empty 15 ml centrifuge tube. Incubate tube in a 37°C water bath.
- ♥ Quickly thaw the cells in a 37°C water bath (about 2 min), and transfer into the 15 ml centrifuge tube containing the wash medium.
- ♥ Centrifuge for 5 minutes at 500×g.
- ♥ Remove the tube from the centrifuge and remove the wash medium by aspiration.
- ♥ Gently resuspend the pellet in 5 ml supplemented Claycomb Medium, and add to the 10 ml of medium already in the T75 flask.
- ♥ Replace the medium with 15 ml of fresh supplemented Claycomb Medium 4 hours later (after cells have attached).

	Vendor	Catalog #
Claycomb Medium	JRH Biosciences	51800
*Fetal Bovine Serum (Lot # 5J0994)	JRH Biosciences	12103-500M
Penicillin-Streptomycin (10^4 U/ml P and 10^4 μg/ml S)	Life Technologies	15140-122
Norepinephrine [(±)-Arterenol]	Sigma	A-0937
L-Ascorbic Acid, Sodium Salt	Sigma	A-7631

L-Glutamine, 200 mM	Life Technologies	25030-081
Trypsin-EDTA, 1×	Life Technologies	25300-054
Trypsin inhibitor, soybean	Life Technologies	17075-029
Fibronectin	Sigma	F-1141
Bacto© Gelatin	Fisher Scientific	DF0143-17-9
Cryovials, Corning	VWR	66021-920
Sterile Acrodisc syringe filters, 0.2 μm	Gelman Sciences	4192
Distilled Water, cell culture grade	Life Technologies	15230-147

*If you have any problems obtaining this lot of serum, please contact:
Ms. Joan Carlson
Research Scientist, JRH
Phone: 913-469-5580 × 287
E-mail: joan.carlson@jrhbio.com
This FBS has been pre-tested by the Claycomb lab for use with HL cells **AND IT IS AN ABSOLUTE REQUIREMNENT THAT THIS PARTICULAR LOT OF SERUM BE USED WHEN CULTURING THESE CELLS. THE CELLS WILL NOT MAINTAIN THE CONTRACTING PHENOTYPE IF THIS FBS IS NOT USED. IT IS ONE OF THE MOST IMPORTANT REAGENTS!!**

BioMEMs Protocols

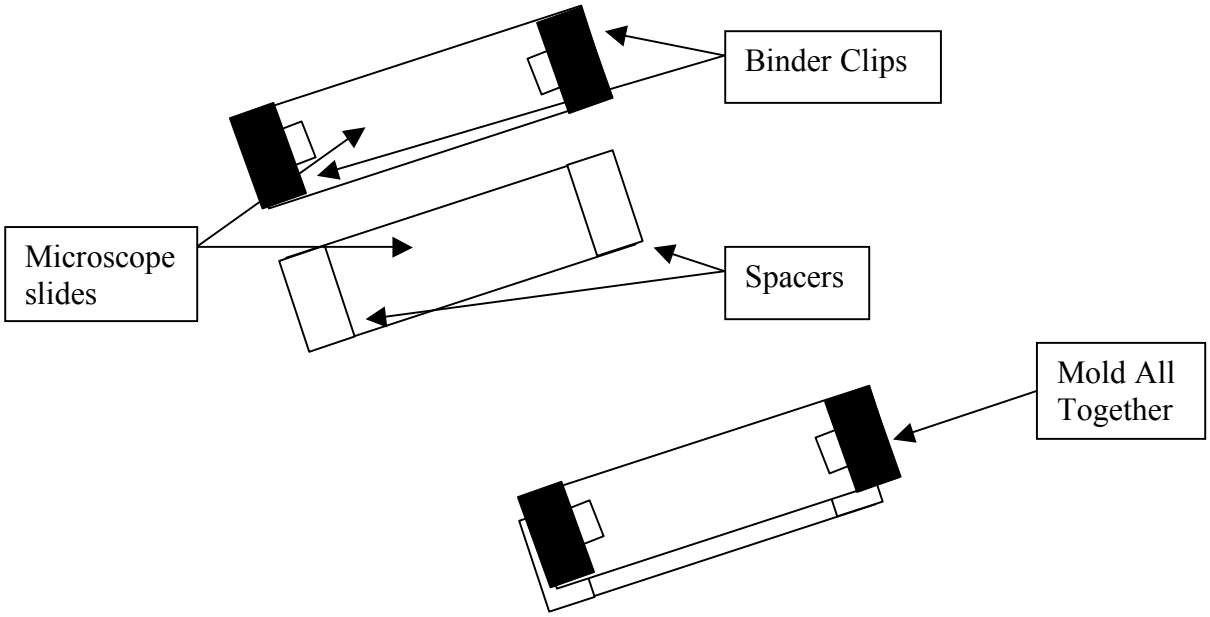
Making 2D Hydrogels

Materials

- Microscope slides
- Glass cutter
- Dye
- Forceps
- Small binder clips
- Monomer formulation with initiator

Procedure

1. Warm up UV light (10 min prior to using)
2. Cut at least two thin pieces from a microscope slide (spacers)
3. Spray down microscope slides, spacers, forceps, dye, and binder clips and place in hood
4. Assemble mold (see picture)
5. Add ~1.5 ml of monomer solution to mold between microscope slides
6. Place on pipette tip box and put under UV light for ~10 minutes
7. Once polymerized, remove clips and pry open the mold taking care not to rip the polymer sheet
8. Using the dye, punch out polymer disks
9. Place disks in 12 or 24-well untreated plates and add sterile PBS
10. Leave plates (with cover off) under UV light overnight
11. Rinse wells with sterile PBS 3x before seeding cells



Photoencapsulation of cells:

Instructions for making 0.6 wt% I2959 stock solution:

Add 50 ml of dH_2O to 0.3 g I2959 in 50 ml conical vial
Heating to 60°C for a few hours may be necessary to dissolve I2959
Wrap vial completely in foil or opaque tape and store @ room temperature
Shelf life of a couple of years

Instructions for making macromer solutions:

Y = g dH_2O ~ ml dH_2O or = g PBS ~ ml PBS
I = g I2959 stock solution ~ ml I2959 stock solution
X = g macromer
W = weight fraction of macromer in polymerization solution

For 0.05 wt% final initiator concentration add the following amounts of Y & I to a measured mass of X:

$$Y = X\left[\frac{11-12*W}{12*W}\right]$$

$$I = \frac{1}{11}(X+Y)$$

Preparation of cells: Spin down cells for 10-12 minutes at 1000-1200 rpm and remove as much media or buffer from cells as possible. Filter macromer solution through a 20 micron filter into conical vial containing cells and resuspend cells by pipetting up and down.

Polymerize for 10 min at ~10 mW/cm^2 (360 nm light, time can be adjusted for more or less intense light source)

Example: Using 0.1 g of PEG-DM to make a 10 wt% hydrogel

X = 0.1 g
W = 0.1
Y = 0.1 g (11-12*(0.1))/(12*0.1) = 0.817 ~ 0.817 ml
I = (1/11)*(X + Y) = 0.083 g ~ 0.083 ml
Total solution volume ~ 0.9 ml
Polymerize under 360 nm light source

Microparticle preparation

1. In a glass tube (15 mm in diameter and 65 mm long) dissolve 0.3 grams of polymer in 2 ml of methylene chloride.

2. While gently vortexing the dissolved polymer solution, add 100 µl of the encapsulant solution drop-wise.

3. Sonicate the solution on ice for 10 seconds.

4. Place the tube on ice. Add 4 ml of a 1.0% polyvinyl alcohol solution slowly (maintain the phase separation).

5. Repeat sonication on ice for 10 seconds.

6. Set up a 400 ml beaker with a stirring rod on a magnetic stir plate. Add 100 ml of 0.3 % polyvinyl alcohol.

7. Pour the emulsion into the center of the stirred solution. Stir for 3 hours.

8. Centrifuge the emulsion and wash the microparticles three times (4000 RPM, 10 minutes, 4oC).

9. After the final rinse re-suspend the microparticles in 5-10 ml of distilled water. Freeze the mixture in liquid nitrogen.

10. Lyophilize the microparticles until they are dry.

Preparing Chitosan Nano/Microparticles

Chitosan nano/microparticles are useful for intracellular delivery of negatively charged molecules and presumably can be prepared with any negatively charged molecule. Here, DNA is used as an example. There are many types of chitosan with different molecular weights and percent deacetylation; results will vary depending on the chitosan used. Experiment can be scaled to prepare larger or smaller batches. Procedure is based on Aral *et al.*, ***STP Pharma Sciences***, **10**: 83-88 (2000) and Berthold *et al.*, ***J. Cont. Rel.***, **39**:17-25 (1996).

1. Dissolve 0.25 wt% chitosan (any type) in 1% acetic acid (takes ~1 day)
2. Prepare solution of negatively charged molecule in water or a dilute buffer. Concentration will depend on the negative charge and solubility of the molecule. For DNA, with 2.8 nmol of negative charge per μg of DNA a concentration of 1 mg/ml (2.8 mM negative charge) in 0.5X TE works well.
3. Put 2 ml of 0.25 wt% chitosan in a small glass vial with a small stir bar. Stir on a stir plate at a speed where sample is almost vortexing.
4. Carefully inject 2 ml of solution containing negatively charged molecule into the stirring chitosan through a 25 gauge needle. Speed of addition affects the initial particle size, but unknown if it will affect the final particle size.
5. Let solution stir for ~3 hours. Stir speed will affect final particle size, but no measurements have been made to characterize the correlation.
6. Centrifuge to separate nanoparticles from solution- 2000x*g* for 10 minutes works well with DNA-chitosan nanoparticles.
7. If desired, supernatant may be saved for assay to determine efficiency of encapsulation.

Protein Patterning using Parylene

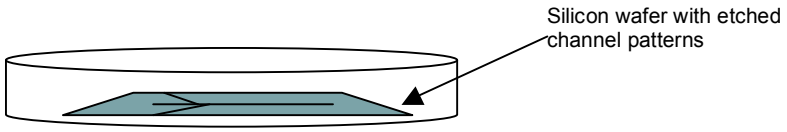
1. Prepare protein solution (usually 20ng/ml of TexasRed-BSA, 50ng/ml of FITC-BSA)
2. Prepare hydrophobic substrate for parylene membrane, either PDMS on glass, polystyrene, or treated glass. For PDMS on glass, clean both surfaces thoroughly before bringing into contact.
3. Add parylene membrane into contact with hydrophobic surface using sharp tweezers.
4. Add protein solution on top of membrane, enough to cover entirely, but not necessary to use more.
5. Incubate for 15 to 30 minutes. It is ok to incubate longer.
6. View with fluorescent microscope.
7. Remove parylene using tweezers, being careful to lift it off rather than drag it.

FOR CO-PATTERNING

8. Add second protein on top of the first protein pattern.
9. Incubate for 15 to 30 minutes, depending on relative strength of the fluorescent signals.
10. View with fluorescent microscope.
    a. View each fluorescent signal separately and merge images.

Protocol for Creating Microfluidic Bioreactors

1. Clean silicon wafer with nitrogen gun



2. Make PDMS
   a. Weigh out silicone elastomer base in a 10:1 ratio by weight against silicone elastomer curing agent in polystyrene cup.
   b. Mix thoroughly.
   c. Degas mixture using vacuum.

3. Pour roughly 40g of PDMS into each dish containing a wafer.

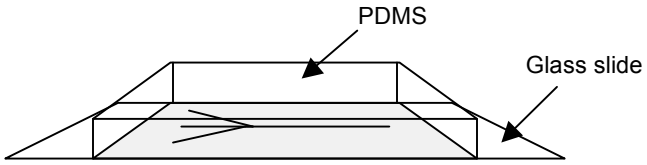
4. Remove all bubble formations using sharp tip.

5. Place in 70°C oven for at least two hours.

6. Cut out PDMS impression avoiding protrusions.

7. Using sharp-tip needle, puncture holes at each inlet and outlet.

8. Plasma clean PDMS and glass slides to bind them irreversibly, and attach as follows:



9. Insert blunt-end needles into holes created at inlets and outlets.

10. Place cylindrical support around needle and fill with epoxy, being careful not to get epoxy on needle tip.

Cell Patterning

Suction Technique

1. Clean PDMS surface with ethanol and water ~ use a tweezer to hold PDMS and dip it in a beaker of ethanol (200 proof) and then in a beaker of distilled water.
2. Dry PDMS with nitrogen air.
3. Dilute 1 mg/ml fibronectin 1:10 with PBS (1X).
4. Coat the surface of a microscope cover slide with the fibronectin solution (dipping slide in solution or spreading solution over slide).
5. Place the PDMS mold on the fibronectin coating of glass slide.
6. Put a drop of boiling 1% aqueous agarose solution along one of the open edges of mold.
7. Apply vacuum suction on the opposite edge ~ fill the channels of PDMS feature with agarose by pulling the solution through the channels with a glass pipet connected to moderate vacuum.
8. Allow agarose solution in the channels to dry for 12 hours.
9. Detach agarose from the PDMS mold.

Stamping Technique

10. Clean PDMS surface with ethanol and water ~ use a tweezer to hold PDMS and dip it in a beaker of ethanol (200 proof) and then in a beaker of distilled water.
11. Dry PDMS with nitrogen air.
12. Dilute 1 mg/ml fibronectin 1:10 with PBS (1X).
13. Coat the features of the PDMS surface with droplets of fibronectin solution (≈ 600 μl) using a micropipet.
14. Let PDMS sit for 15 minutes to dry.
15. Remove the excess fibronectin solution on PDMS with vacuum suction.
16. Clean PDMS surface with PBS and water. Use a tweezer to hold PDMS and dip it in a beaker of PBS (1X) and then in a beaker of distilled water.
17. Dry PDMS with nitrogen air.
18. Use the feature surface of PDMS and stamp it on a microscope cover slide (placed in a 6-well plate).
19. Cover the cover slide with cell culture (≈ 2 ml in each well of a 6-well plate).

Making gradient solution using microfluidic device

Note: Steps 1 through 4 need to be performed in the clean box.

2. Cut the PDMS stamp with razor blade out of the Si Wafer (be careful when cutting not to break the Si wafer)
3. Place PDMS stamp with microfluidic pattern facing up in the box
   Note: at this time you should refill the petri dish with PDMS to avoid exposure of the Si wafer to air contamination.
4. Punch hole into the inlet (from the patterned side to the non patterned side) with pipetting 18G needle.
   a. Cut the end of the PDMS stamp so that the outlet channel is open to the air when placed on the glass slide
5. This step is to achieve an irreversible seal between the glass slide and the stamp.
   a. Put the PDMS stamp and a glass slide cleaned with ethanol and with no dust in the chamber.
   b. Set the power to medium, turn on the plasma etcher and then turn on the vacuum pump (the vacuum pump isn't turned on at the beginning because we don't want a high vacuum that would result in a strong plasma etching).
   c. Stop the pump and the plasma etcher 30-40s after the plasma has formed (pink/violet glow).
   d. Assemble the 2 surfaces and apply some pressure (with back of a needle for example) on the PDMS stamp.
6. Cut with a blade 3 pieces of tubing around 10cm each
7. Prepare the epoxy glue. Use a 9" Pasteur pipette to mix the 2 components.
8. Place the tubing in the inlets and seal with the epoxy glue using the Pasteur pipette.
9. Let the glue dry for around 10 min.
10. Place microfluidic device on microscope stage
11. Fill in Hamilton gastight syringes (0.5mL or 1mL…) with the appropriate solutions
12. Place syringes in pump rack and align manually the plungers
13. Adjust height of the pump with the jack so that the tubing and the syringes are at the same level
14. Connect the tubing to the needles.
15. Set flow rate at 50μL/min and flow solution in the tubing until it reaches 1cm before the inlets.
16. Set the flow rate at 5μL/min 1cm before the inlets and let the solution flow until gradient establishes in the outlet channel.
17. To get rid of the bubbles in the outlet channel, tap gently on the outlet channel or increase the flow rate.

Source of Chemicals for Tube manufacture

EDMA - Ethylene (glycol) dimethacrylate (crosslinking agent); Sigma-Aldrich 33,568-1, 98% purity, 5 or 100ml quantities. Store in refrigerator.

HEMA - 2-hydroxyethyl methacrylate (HEMA) (monomer); Sigma-Aldrich 47,702-8, 99+% purity, 100 ml quantities. *This needs to be spiked with EDMA before use. To a full bottle of HEMA, add 100 ml of EDMA (spiked to 0.1% EDMA).* Store in refrigerator.

MMA – methyl methacrylate (hydrophobic monomer); Sigma-Aldrich M5,590-9, 99% purity, 25ml quantities. This may or may not be spiked with EDMA – formulations (except SFX 205) are not spiked wit EDMA. Store in refrigerator.

APS – ammonium persulfate (initiator); Sigma-Aldrich 43,153-2, 99.99+% purity, 50g quantities. Separate initiator into many aliquots (in "red" cryogenic vials), and store in refrigerator.

SMBS – sodium metabisulphite (accelerator); Sigma-Aldrich 25,555-6, 97% purity, 100g quantities. Store at room temperature.

TEMED – N,N,N',N'-tetramethylethylenediamine (accelerator); Sigma-Aldrich T8133, 99+% purity, 50 ml quantities. Store in refrigerator.

Tube Manufacture

1 mold is assembled.

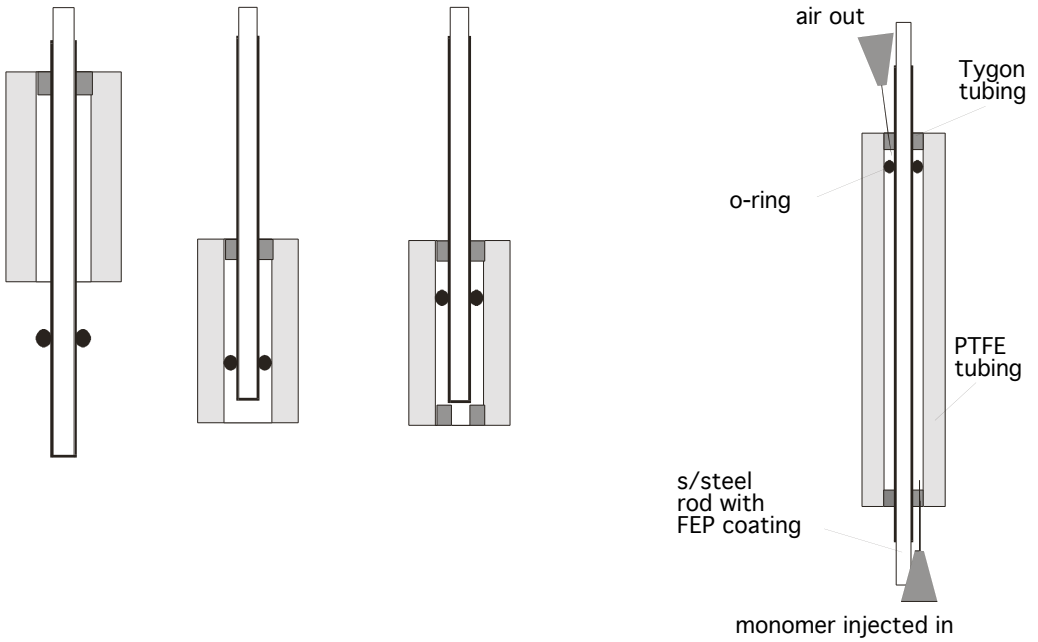
You should have 2 spare o-rings, spare (long) PTFE tubing, tygon tubing (which needs to be cut and dispensed with each mold)

1. Cut Tygon tubing into two small pieces
2. Insert one piece of tygon tubing into the end of the PTFE tube.
3. If not in place already (I think they are), put o-ring into the groove cut on the FEP-coated stainless steel tube.
4. Insert the s/steel tube (o-ring close to you) into the PTFE tube and place tube through the hole in the tygon tubing (which should be at the far end)**(Fig)**.
5. Push the s/steel rod into the PTFE tube fully. A large length of this rod is now sitting on the other side, supported by the tygon tubing **(Fig)** .
6. Place the other tygon tubing piece into other end, and then push the FEP rod through this. Have the o-ring sitting close to the tubing, with the FEP-coating exposed on the outside at both ends **(Fig)**.
7. Using two 30-gauge needles, push one through the tygon tubing near the o-ring to permit air to escape, and attach the other needle to your monomer mixture **(Fig)**.
8. Holding the mold upright, inject the monomer so that all air is removed.
9. Remove the top needle, then the lower one, still injecting so that air doesn't come in to replace the volume of the needle tip as it is removed.
10. The cast tube will be of similar dimensions to the tygon tubing.

To remove:

1. Use tweezers to pull off the tygon tubing from both ends.
2. Pull from the non o-ring end to remove the cast tube.
3. Either leave on or pull off the FEP-coated s/steel tube.

Purposes:
O-ring: To assist in removal of the cast tube from the mold. Pull from the non o-ring end to remove the cast tube.
Tygon tubing: To maintain symmetry of the central rod, and the resulting cast material. Also allows injection through it.
PTFE tube: ID forms the OD of the cast tube. Should release the cast tube easily.
FEP-coated s/steel tube. FEP-coating permits the easy release of the cast tube – s/steel provides rigidity and straightness for symmetrical casting of a tube. **Don't damage the FEP-surface.**



Monomer composition:

There has been no morphological investigation into the nature of the porous coating of such cast tubes, only that a white material forms over the tube, and is bound to it.

Monomer Composition:

35wt% H_2O
65wt% HEMA
0.25% EDMA
0.5% APS
0.5% TEMED

Note: This will polymerize **FAST.** Only do one mold per monomer mix. Suggest splitting your monomer mixture into the number of molds you will have (ie. 4 molds, split into four) *after* you add the APS. In this instance, the TEMED is added last, and you have 3-4 minutes to get it into the mold. **Do this one mold per monomer mix.**

1. Prepare the mold ready for injection.
2. Add the TEMED last – start a stopwatch now!!
3. Cast for 20 minutes (from when the TEMED was added). You may wish to vary this time to get different effects.
4. Remove from mold, and add (with FEP-coated s/steel tube still attached) to H_2O, or a 98:2 ratio of H_2O: HEMA. Once again this ratio can be varied. Different effects will be seen.
5. After one hour place in water, or exchange water present.
6. After 5 hours remove the tube and take off the FEP-coated s/steel tube.
7. Soxhlet/treat as any device.

Clean components after each use (soak in water) Do not roughen the FEP-coating. Use new PTFE tubing if release becomes difficult.

Procedure for manufacturing two hydrogel tubes.

1. Remove 2-hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) from fridge and sit on bench at for at least 1 hour (to warm up to RT).
2. Weigh out 0.1 to 0.15 g of ammonium persulfate (APS-fridge) into a vial, and top up with water to make 10-wt% solution (do not tare balance). LABEL vial APS.
3. Weigh out 0.1 to 0.15 g of sodium metabisulfite (SMBS-bench) into a vial, and top up with water to make 10-wt% solution (do not tare balance). LABEL vial SMBS.
4. Prepare glass mold by cutting glass tube of required diameter into required length (typically 10-15 cm), blowing it out with filtered air, and sealing both ends with rubber septa.
    a. (Optional) If tube needs to be silane-modified, treat glass in appropriate manner (see surface modification SOP.
5. Dispense appropriate volumes of HEMA, MMA and water into vial and swirl until solution is clear.
6. Lie out two syringes (usually 3 or 5 ml), filter, filter adapter and four 20G needles on the bench. Attach filter and adapter on one syringe, and a needle onto the other syringe.
7. Puncture one septa of each glass mold with a needle and leave them in there in there.
8. Add volume of APS to the monomer solution and swirl until clear.
9. Apply vacuum to the monomer mixture vial and remove dissolved gas from monomer mixture (2 minutes).
10. Add volume of SMBS to the monomer solution and swirl until clear. Polymerization has now started
11. Slowly withdraw monomer mixture into the syringe, taking care not to introduce bubbles.
12. Remove needle and push through filter into second syringe.
13. Remove the filter and adapter, and place fresh needle onto syringe
14. Inject into glass molds, taking care not to leave any bubbles in the glass mold.
15. Place in chuck of stirrer at appropriate speed. Ensure chuck is firm on the glass mold.

Procedure for Preparing Devices for Implantation.

1. Manufacture hydrogel tubes as outlined in protocol
2. Switch off stirrer and remove glass molds from drill chuck. Spinning is generally maintained overnight (preferable), or six hours.
3. Remove rubber septa, and tilt to drain water out of mold. Score with the glasscutter either 1/3 or 2/3 across length, and break the glass mold without breaking soft tube inside.
4. Pull apart the glass tubing slowly, and work the tube end out of the mold.
5. Score again (in the middle) and remove the glass mold. If necessary, use tweezers on ending of hydrogel tube to pull the mold off the hydrogel tube.
6. Cut 5mm off ends and transfer to filtered deionized water in 5ml or 50 ml centrifuge vial. Cut out any imperfections in the hydrogel tube. Let stand for 10 minutes.
7. Under the stereomicroscope (at magnification 1.0), cut (with a razor) hydrogel tubes into x mm - length vary depending on required nerve guide – see other SOP.
8. Place in scintillation vial containing filtered deionized water.
9. After cutting is finished, place tubes in histology case and place this in Sohxlet extractor.
10. Extract for desired time (from 2 hours to overnight).
11. Under a sterile hood, place nerve guides in scintillation vial in PBS, and select only well-formed tubes, placing them into individual cryogenic vials.
12. Add filtered PBS solution to cryogenic vials (about 2/3 full), and screw on the lids.
13. Puncture and leave a 27G needle into the cap.
14. 14.Place vials into a sterilization package and seal.
15. Autoclave at 120ºC for 20 minutes. Sterilize only – do not sterilize and dry (this will melt vials).

Chemistry/Synthesis Protocols

Making Alginate Particles

1. Prepare a 2.5% solution of sodium alginate in water
2. Autoclave solution or heat in oven to dissolve alginate (should be yellow and clear)
3. Mix 20.6 mg calcium carbonate and 74 mg D-glucono-δ-lactone in 1 ml of distilled water (will not dissolve)
4. Add 1 ml of calcium carbonate/GDL solution to 4 ml of alginate solution in a plastic scintillation vial
5. Cap the vial and let solution polymerize overnight
6. After polymerizing overnight, scrap solution into a mortar (largest one available).
7. Flash-freeze the polymer with liquid nitrogen and use the pestle to crush up the polymer
8. Add the resulting fine particles to an eppendorf tube and immediately place in the -80 ºC freezer
9. Freeze-dry

Synthesis of Acr-PEG-Peptide/Growth Factor

1. Determine how much Acr-PEG-stuff you want/need and convert to moles
2. Calculate needed amount of PEG(3400)-NHS (mol to g – MW ~3220)
3. Determine how much of the pendant group to add – use 20% excess
4. Prepare either a 50 mM Tris buffer, pH 8.5 or a 50 mM sodium bicarbonate buffer, pH 8.4
5. React PEG-NHS and pendant group in ~100 ml of buffer in a round bottom flask for 2 hours
6. Pour liquid into a freeze-drying vessel and freeze in -80 ºC
7. Freeze-dry
8. Resuspend in PBS and dialyze in appropriately pore sized dialyzer for 3 days with 2 exchanges of PBS
9. Freeze the dialyzed product again at -80 ºC
10. Freeze-dry

Digestion of Constructs

Papain Digestion Solutions

1. PBE buffer - Phosphate (100mM)(40%diNa;60%monoNa), EDTA (10mM), pH 6.5

   Add 7.1g Na_2HPO_4, 1.86g Na_2EDTA to 500ml dH_2O
   (Na_2HPO_4 142 g/mol, $Na_2EDTA.H_2O$, 372 g/mol)
   Adjust to pH 6.5 with conc. HCl
   Filter sterilize
   Store at 4°C

2. Papainase (type III, 19U/mg) (Worthington #P-3126)

   Make fresh: papain (125 g/ml), cysteine (10mM*), phos(100mM), EDTA (10mM), pH 6.3 *MW cysteine-HCl =175.6g/mol

   Dissolve 0.035g cys in 20ml PBE
   Filter Sterilize
   Add 0.10ml sterile papain stock (25mg/ml)
   (use TB syringe and needle; alcohol wipe stock enzyme)

Proteinase K Digestion Solutions

1. Stock solution TRIS, EDTA

   To make one liter: 6.055g TRIS (50mM) in ~950 ml H_2O)
   Adjust pH w/ 6N HCl to 7.6
   Add 0.372 g EDTA x 2 H_2O (1mM)
   Add H_2O up to 1000 ml

2. Solution A (working solution)

   100ml from TRIS, EDTA stock
   add 18.5 mg iodoacetamide (1mM)
   add 1 mg pepstatin A (10 g/ml)
   heat to dissolve –takes a while

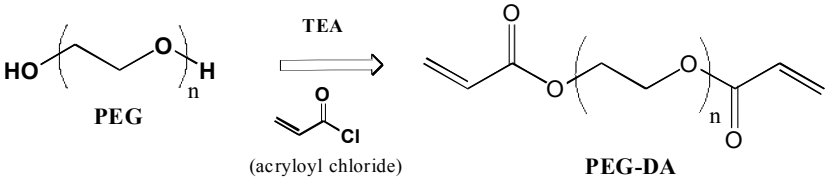
3. Solution B (working solution)

   50ml from solution A
   add 50 mg of proteinase K (1mg/ml)
   incubate in 56°C water bath overnight (~16hr)

Digestion

1. Weigh wet constructs
2. Freeze dry constructs
3. Weigh dry constructs
4. Add digestion solution to vials containing constructs
    a. 1 ml for 2mm thick sample
    b. 2 ml for 5mm thick sample
5. Incubate in 60°C water bath for 10 to 16 hrs
6. Vortex
7. Refreeze until ready for assays

Synthesis of Diacrylated/Dimethacrylated PEG

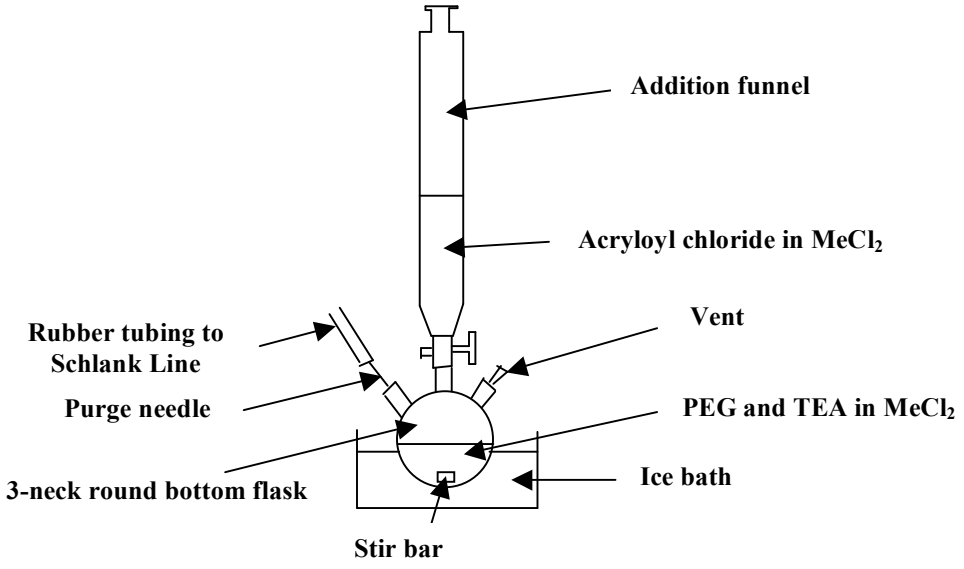


Important note in this synthesis: It is very important that all glassware, tubing, etc., in this synthesis is kept as dry as possible. I usually dry all glassware in the 80C oven prior to the synthesis.

Another note: if PEG is to be *methacrylated*, use a molecular weight of 104.5 g/mol and density of 1.07 g/ml, as well as a different purity (depending upon company – usually written on the bottle) in the calculations in step 7 below.

This protocol is for the synthesis of 10 grams of diacrylated product.

1) Obtain a dry, 3-neck, round-bottom flask (250 ml).
2) Place 10 grams of poly(ethylene glycol) (PEG) into the flask. Various molecular weights are available, the most common being PEG3400 and PEG4600.
3) Add 30 ml methylene chloride to the flask, and set up the apparatus as depicted below:



4) Calculate the amount of triethylamine (TEA) required. Usually, 12 moles of TEA are added per mole of PEG. For a ten-gram synthesis of PEG3400DA, this is 4.92 ml. In general, to calculate amount of TEA required, use the following formula (note that different molar ratios of TEA to PEG can be used):

   Amt. TEA = (10 g PEG)/(3400 g/mol PEG)*(12 mol TEA/mol PEG)*(101.2 g/mol TEA)/(0.726 g/ml)

5) Before adding the triethylamine, it is necessary to purge the flask to remove any water vapor that may be present. To do this, connect an argon purge needle to one of the necks of the flask using a rubber septum. The purge needle tip should be submerged in the solution. On the third neck of the flask, place a septum with a vent needle (see Figure above). Purge the flask for 5 minutes
6) Add the appropriate amount of TEA (4.92 ml for 10-g synthesis).
7) In the addition funnel, combine 15 ml of $MeCl_2$ with acryloyl chloride (AC). Usually 10 moles of AC are added per mole of PEG. To calculate the amount of acryloyl chloride to add, use the following formula (note that different molar ratios of acryloyl chloride to PEG can be used):

   Vol. AC = (10 g PEG)/(3400 g/mol)*(10 mol AC)/(mol PEG)*(90.5 g/mol AC)/(0.96 purity)/(1.114 g/ml)

   For a 10-g synthesis, this amount is 2.50 ml.

8) Make sure the apparatus is setup as shown in the Figure above. It is very important to make sure the flask sits in an ice bath and make sure that the ice bath remains ice-cold during the duration of the following step.
9) Very <u>SLOWLY,</u> drip the acryloyl chloride solution into the PEG/$MeCl_2$/TEA solution. When I say very slowly, this means that there should be between 5-15 seconds between each drop! The solution will turn yellow or orange if the solution is added too quickly (the solution will actually probably turn light yellow regardless).
10) Let the acryloyl chloride solution drip for 45-60 minutes, then cap the flask and place on a stir plate in the cold room (4C) and stir overnight.

    Note: it is not uncommon for the solution to be colored, even fairly red colored. This is caused by the formation of small byproducts in the reaction mixture. Through purification procedures (like washing with water or dialysis), the color can be removed.

11) The following day, the product is precipitated in ethyl ether. Instead of precipitating the entire product (and wasting time and expensive ethyl ether), I usually will precipitate maybe a quarter of the product and verify substitution using NMR. To do this, I precipitate 10-15 ml into a beaker containing a stir bar and 200-300 ml ethyl ether (on ice). The precipitate can then be filtered using

Whatman paper and a ceramic filter, and the product is placed in a watch glass or glass Petri dish for drying in the vacuum oven at 50C for several hours.

12) Once the product has been dried, it can be evaluated for ability to polymerize and can be characterized using NMR.

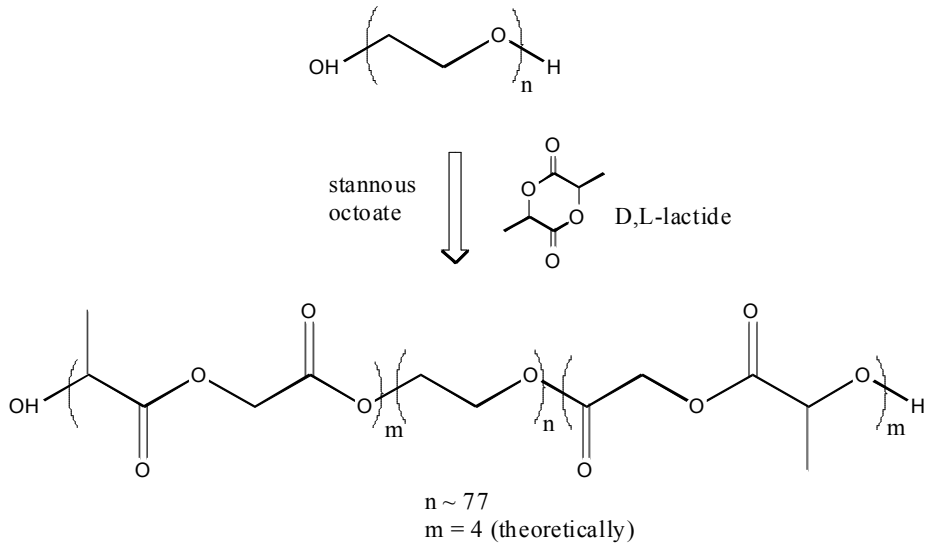
    Note: if the substitution is low, the product can be re-acrylated or remethacrylated.

13) If substitution/ability to polymerize is satisfactory, the remainder of the product can be precipitated. Usually, a 10:1 ratio of ether:product is used for precipitation. Keep in mind that ethyl ether is expensive and also must be disposed of through EH&S, so try to minimize waste generated through judicious experimental design.
14) Clean up your mess.

Addition of Lactides to PEG

Note: it is very important to keep this reaction as dry as possible. I usually dry all glassware in the 80C oven before starting.

The following procedure is for addition of 2 lactide molecules per side of poly(ethylene glycol) of molecular weight 3400 (PEG3400):



1) Place 10 g PEG into a 25-50 ml round bottom flask. Add a stir bar.
2) Add D,L-lactides (Polysciences). Depending upon PEG molecular weight used and degree of substitution desired, the amount is calculated using the following formula:

   Amt. of lactides = (10 g PEG)/(3400 g/mol)*(4 mol lactides/mol PEG)*(144 g/mol lactides)

   For placing one lactide group on each end of PEG3400, this amount is 1.694 g.

3) Purge the round bottom flask with argon or nitrogen for 5-10 minutes (make sure the flask is vented with a vent needle), then add stannous octoate (1:200 mass ratio to PEG), which acts as a catalyst for the reaction. Stannous octoate has a density of 1.25 g/ml and is also known as 2-ethylhexanoate. For our case, the calculation is shown below:

   Amt. stann. oct. = (1/200)*(10 g PEG) = 50 mg stannous octoate (40 µl)

4) Heat the reaction to 140C using a temperature-controlled hot plate and an oil bath. It is very important that the top of the round bottom flask remains submerged in oil, so try to use the smallest flask possible. When heated, the lactides will

vaporize to some extent and condense/crystallize on the top of the round bottom flask if the top is not submerged in oil.

5) Allow the reaction to proceed for 4 hours at 140C, then place the reaction in a refrigerator until it is needed.
6) The product must now be purified. If it is to be acrylated or methacrylated, it is very important that all stannous octoate is removed. Any remaining stannous octoate will inhibit the acrylation/methacrylation steps. PEG-LA can be purified by dissolving in a minimum amount of methylene chloride (25 ml for our case) and precipitating (either dripping from a separatory funnel or dropwise using a glass pipet) into ice-cold ethyl ether (10:1 ether:product by volume). The product is then filtered through Whatman paper using a ceramic filter and dried in a vacuum oven at 50C anywhere from several hours to overnight (depending upon purity desired). This dried product can be re-dissolved in a minimum amount of methylene chloride and re-precipitated in ethyl ether to further purify the product and separate out the stannous octoate. For best purification, the product should be precipitated a total of 3 times.

   *Note: before precipitating and/or re-precipitating the entire product, it is advisable to purify a small sample and analyze using NMR to make sure substitution is okay. Ethyl ether is expensive and it's important to minimize generation of hazardous wastes.

   **If the PEG-LA product is to be acrylated or methacrylated, the procedure for acrylating/methacrylating PEG can be used with a few small adjustments. Mainly, the calculations must be altered because now the molecular weight of the PEG molecules has changed slightly (e.g., for PEG3400-DA with two lactide molecules per side the molecular weight has changed from 3400 to 3400+144*4 = 3976 g/mol). The rest of the procedure is the same, though.

7) Clean up your mess.

Acryloyl-PEG-peptide preparation

Materials:

Peptides conjugated to monoacrylated PEG
acryloyl-PEG-n-hydroxysuccinimide (3400 Da; Nektar Therapeutics, Birmingham, AL)
50 mM sodium bicarbonate buffer

Procedure:

1) Peptides were conjugated to monoacrylated PEG as previously described
2) Briefly, peptides were combined with acryloyl-PEG-n-hydroxysuccinimide in 50 mM sodium bicarbonate buffer (pH 8.5)
3) Reaction allowed to take place at room temperature for 2 h.
4) The resulting acryloyl-PEG-peptides were lyophilized and stored at -20°C until use.

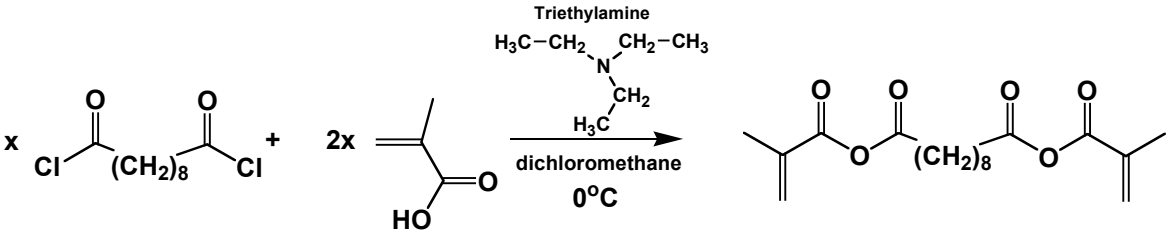
How to prepare an X% PEG-DA Solution

1. Weigh a sterile 15 mL conical vial and write the weight on the vial
2. In the laminar flow hood, add the amount of PEG-DA you want to use to the 15 mL conical vial.
3. Weigh the conical vial again, noting the difference as the PEG-DA weight.
4. Divide the polymer weight by 0.X to get the total solution weight that you will make.
5. Subtract the polymer weight from the total solution weight to get the amount of water/initiator that you will add
6. Calculate amount of initiator solution that you need to add based off of the stock initiator solution concentration and the final initiator concentration that you want (a $M_iV_i = M_fV_f$ type equation works fine here, substituting initial and final masses for volumes)
7. Subtract the amount of initiator solution from the amount of water/initiator required that was calculated in step 5 to get the amount of deionized water that you will add.
8. Add this amount of water to the PEG-DA in the conical vial, dissolve by vortexing, filter sterilize
9. If you are encapsulating cells, add the PEG-DA solution to a cell pellet that will give the desired cell concentration, suspend the cells by repeated pipetting.
10. Add the required amount of initiator solution (filter sterilize if it is not already sterile), mix by repeated pipetting.
11. Polymerize PEG-DA solution

Methacrylated Sebacic Acid (MSA) Preparation[1]

[1]See Tarcha, et al., *J. Polym. Sci, Part A, Polym. Chem.* **2001**, *39*, 4189.

Synthesis.
Overall reaction:



1) Determine the amounts of reagents. Use 2.2 equivalents of methacrylic acid (MAA) and triethylamine (TEA) relative to the moles of sebacyl chloride (SbCl) you will be using.
2) Add MAA to a 3-neck round bottom flask (of the appropriate size) fitted with an addition funnel on the center neck and containing a stir bar.
3) Place the set-up on top of a stir plate in a dish that will accommodate an ice bath.
3) Dilute the MAA with approximately 20 times the amount of methylene chloride (MeCl) as MAA (e.g. 30 ml MAA in 600 ml MeCl).
4) Add the TEA, cover the remaining necks with rubber septa, fill the dish with ice and chill the flask for 1 hour, with stirring.
5) Add the SbCl to the addition funnel along with an equal amount of MeCl. Cover the opening with a rubber septa.
6) Add the SbCl to MAA solution dropwise over about 1-2 hours depending on the scale (e.g. 40 g of SbCl should take 2 hours).
7) Once addition is complete, stir for 3 hours while maintaining the ice bath.

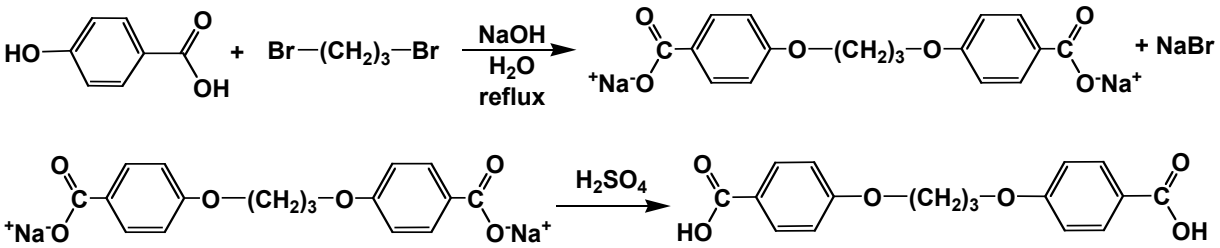
Purification.
1) Remove any visible triethylamine-hydrochloride salts via vacuum filtration.
2) Remove any remaining salts by extracting the organic layer with 2 washes of saturated sodium bicarbonate followed by 2 washes with deionized water. The volume of the aqueous layer should be about equal to that of the organic layer (e.g. 600 ml of MeCl = 600 ml of water). This is often easier if you separate large reactions into 2 smaller portions for the extraction steps.
3) Collect (and combine if 2 batches) the organic layer and dry over sodium sulfate.
4) Remove the drying agent via gravity filtration.
5) PRIOR TO ANY FURTHER STEPS - add some type of inhibitor. This will prevent significant oligomerization of the MSA. The monomer itself will be of a honey-like consistency if no oligomerization occurs – the more oligomerization, the more solidified the monomer becomes, potentially complicating usage. Good inhibitors include 2+3-*t*-butyl-4-methoxy-phenol or if cellular compatibility is desirable, Vitamin D can also be used.
6) After addition of the inhibitor, use a rotovap to remove the MeCl, preferably at 0°C using a strong vacuum to minimize the chances of oligomerization.

7) The MSA can be characterized via NMR using chloroform-*d* as the solvent. The methacrylation efficiency can be calculated based on the ratio of the integrals for the vinyl resonances to that for those next to the acetate protons. Common %methacrylation = 90-95%.

1,3-*Bis*(*p*-carboxyphenoxy) propane/hexane (CPP or CPH) Preparation

Synthesis.
NOTE: The diacid molecules are very stable, so a large scale reaction is described. Adjust reagent amounts accordingly if you want to work on a smaller scale. Although the synthesis described is for CPP, the steps are identical for CPH. Simply use 1,6-dibromohexane instead of 1,3-dibromopropane in step 7.

Overall reactions:



1) Place 152 g (1.1 mole) of 4-hydroxybenzoic acid (HBA) into a 1000 ml 3-neck round bottom flask.
2) Dissolve 110 g (2.75 moles) of NaOH in 400 ml of DI-H_2O to prepare a 6.9 M solution. This large excess is needed to ensure deprotonation of both sites on the HBA. (**CAUTION**: this will get HOT – dissolve the NaOH in stages and/or chill the solution. Do not use more than the stated 400 ml of water. Once you add this to the flask and begin stirring, it will essentially be at maximum capacity).
3) Place the 3-neck flask in a heating mantle. Fit one of the necks with a condenser (making sure to grease the joint) and the center neck with an overhead stirrer (see Figure)
4) Turn on the water to the condenser. Pour the NaOH solution into the flask slowly and begin stirring to dissolve the HBA.
5) Fit the remaining neck with an addition funnel with at least a 60 ml capacity, again greasing the joint to ensure a good seal (see Figure).

6) Begin heating the solution until a gentle reflux is reached.
7) Put 50.8 ml (0.5 moles) of 1,3-dibromopropane (DBP) into the addition funnel. Cover the opening with a rubber septa. **NOTE**: If you want CPH use 77.2 ml (0.5 moles) of 1,6-dibromohexane instead of DBP.
8) Maintaining the reflux, add the DBP slowly – about 8-10 drops/minute.
9) Once all the DBP has been added, reflux the solution overnight. After the reflux, turn off the heat and cool the solution to room temperature.

At some point in the reaction, the solution should become heterogeneous and white precipitate will be obvious. This may occur once all the DBP has been added or not until the solution is cool and it crashes out.

Work-up.
1) Filter the solution and collect the precipitate.
2) Put the collected solid in a 2000 ml beaker and wash with ~ 1000 ml of methanol. This will remove the NaBr salts that formed during the reaction.
3) Filter the solution and collect the precipitate. Keep a small amount to analyze by NMR.
4) Put the collected solid in a 3000 or 4000 ml vessel. Set up the overhead stirrer. Add about 1500 ml of DI-H_2O and stir to dissolve the solid.
5) Continue the stirring and begin adding sulfuric acid. Precipitate should form almost immediately. Continue adding sulfuric acid until the consistency of the mixture becomes like cottage cheese. If stirring becomes very difficult, add more water.
6) Once no more solid crashes out, filter the solution and collect the precipitate. Lyophilize to dry. Common yield is about 50%.
7) NMR analysis can be performed on the salt form (kept from step 3) using D_2O as the solvent. Comparison of the integrals for the aromatic protons to those for the central propyl group will provide the overall % substitution achieved.

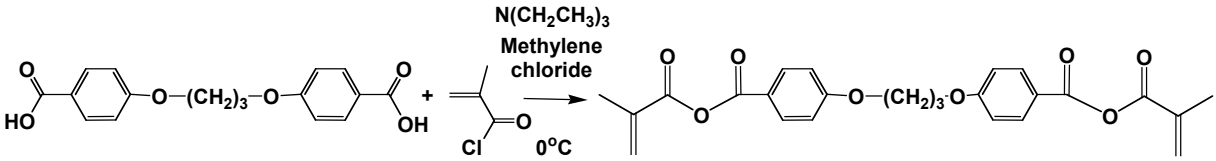
(1,3-*Bis*(*p*-carboxyphenoxy))propyl/hexyl Dimethacrylate[1]

[1]See Tarcha, et al., *J. Polym. Sci, Part A, Polym. Chem.* **2001**, *39*, 4189.

Synthesis.

NOTE: Although the synthesis described is for (1,3-*bis*(*p*-carboxyphenoxy))propyl dimethacrylate (CPPDM), the steps are identical for CPHDM. Simply use 1,3-*bis*(*p*-carboxyphenoxy)hexane instead of 1,3-*bis*(*p*-carboxyphenoxy) propane in step 2.

Overall reaction:



1) Determine the amounts of reagents. Use 2.2 equivalents of methacryloyl chloride (MACl) and triethylamine (TEA) relative to the moles of 1,3-*bis*(*p*-carboxyphenoxy) propane (CPP) you will be using.
2) Add CPP to a 3-neck round bottom flask (of the appropriate size) fitted with an addition funnel on the center neck and containing a stir bar. Cover the addition funnel with a rubber septa. **NOTE**: If you want CPHDM, use 1,3-*bis*(*p*-carboxyphenoxy) hexane for this step.
3) Place the set-up on top of a stir plate in a dish that will accommodate an ice bath.
3) Dilute the CPP with approximately 10 times the amount of methylene chloride (MeCl) as CPP (e.g. 15 g CPP in 150 ml MeCl). The CPP will not dissolve. The solution will look like a suspension.
4) Fill the dish with ice to chill the reaction.
5) Add the TEA and cover the remaining necks with rubber septa. Bubble with inert gas for 1 hour (argon or nitrogen) while stirring at 0°C. The majority of the CPP should dissolve at this point, leaving a slightly heterogeneous, possibly light brown solution.
6) Remove the bubbling gas line. Add the MACl to the addition funnel via a syringe.
7) Add the MACl to the CPP solution dropwise over about 1 hour, depending on the scale.
7) Once addition is complete, stir for 4 hours while maintaining the ice bath.

Purification.

1) Remove any visible triethylamine-hydrochloride salts via vacuum filtration.
2) Remove any remaining salts by extracting the organic layer with 2 washes of saturated sodium bicarbonate followed by 2 washes with deionized water. The volume of the aqueous layer should be about equal to that of the organic layer (e.g. 250 ml of MeCl = 250 ml of water).
3) Collect the organic layer and dry over sodium sulfate.
4) Remove the drying agent via gravity filtration.
5) PRIOR TO ANY FURTHER STEPS - add some type of inhibitor. This will prevent significant oligomerization of the anhydride monomer. Good inhibitors include 2+3-*t*-

butyl-4-methoxy-phenol or if cellular compatibility is desirable, Vitamin D can also be used.
6) After addition of the inhibitor, use a rotovap to remove the MeCl, preferably at 0°C using a strong vacuum to minimize the chances of oligomerization.
7) The CPPDM can be characterized via NMR using chloroform-*d* as the solvent. The methacrylation efficiency can be calculated based on the ratio of the integrals for the vinyl resonances to those for the aromatic protons. Common % methacrylation = 90-95%.

HA synthesis

Materials:

HA (MW 50 kDa from Lifecore)
Methacrylic anhydride
5N NaOH (to adjust pH)
Dialysis materials

Procedure:

1) Add HA at 1 wt% to deionized water
2) Adjust the pH to 8
   i. put this in a multineck round bottom flask with 100 or 200 ml of the solution and put a pH probe in while the solution is stirring
3) Add the methacrylic anhydride (about 2 ml per 200 ml of the HA solution) slowly to the solution
   ii. As the methacrylic anhydride is added, there will be quite a bit of fluctuation in the pH
4) Adjust the pH intermittently to maintain a pH of 8
5) Leave on ice for several hours while you monitor every 15 minutes or so and then if the pH seems to be stable
6) Place in the cold room stirring overnight
7) Dialyze (at least 2 days with several changes of water)
8) Lyophilize (takes a few days due to the large volume)
9) Store frozen in the powder form.

Methacrylation of Hyaluronic Acid (HA)

Materials
hyaluronic acid from streptomyces equi. (Fluka)
methacrylic anhydride (Aldrich)
5 N NaOH
95% EtOH
dialysis supplies

Methods
1. Make 1% solution (w/v) of HA in diH_2O. Heat at 60°C with stirring until dissolved. Solution will be very viscous. Let cool to room temp. before proceeding with reaction.
2. In cold room, add 10-fold molar excess methacrylic anhydride dropwise to HA solution.
3. Maintain pH at 8-9. This will require frequent addition of 5 N NaOH to the reaction mixture over several hours. Allow reaction to occur on ice.
4. Let react at 4°C overnight with stirring.
5. Precipitate methacrylated HA (HA-MA) into chilled 95% EtOH (10-fold volume ratio of EtOH:HA solution).
6. Resulting precipitate is white and gooey. If desired, reprecipitation may be done.
7. Dissolve precipitated HA-MA in diH_2O.
8. Add HA-MA solution to dialysis tubing (MWCO 10,000) and dialyze against several changes of diH_2O for 2 days.
9. Lyophilize and analyze via NMR.

Modified Protocol for making Bovine Fibrin (Sigma)

(Modified from Tranquilo)

→ Make Under Sterile Conditions ←

Fibrinogen (Fluka #46312 > 70% protein content)
Thrombin (Sigma #T4648 70 NIH units/mg)

Fibrinogen

1. Warm 108 mL PBS to 37°C.
2. Add 1.08 (g) of fibrinogen (slowly sprinkle on top)
   (gently agitate, do not vortex or stir)
3. Filter through a 0.20 μm syringe filter
4. Distribute into **3.0 ml** aliquots.
5. Store at –80°C.

Thrombin

1. Dissolve 0.0064g of thrombin powder (450 NIH units) in 18 mL of PBS to yield a solution of 25 NIH units/ml.
2. Filter through a 0.20-um syringe filter
3. Distribute into **0.5 ml** aliquots.
4. Store at –80°C.

Another Modified Protocol for making Bovine Fibrin (Sigma)

Source
Fibrinogen (Fluka #46312 > 70% protein content)
Thrombin (Sigma #T4648 52 NIH units/mg)

NEED:

Aprotinin: **225 KIU/ml**
Fibrinogen: **3 mg/ml**
Thrombin: **1.25 NIH Units/ml**
$CaCl_2$ **20 µmol**

STOCK SOLUTIONS:

Aprotinin: **2250 KIU/ml** → **Already Made**
Fibrinogen: **10 mg/ml** → **Add 0.45 g in 45 ml**
Thrombin: **150 NIH/ml** → **Add 14.4225 mg (750 NIH units) in 5 ml**
CaCl2: **0.000680180 mol/ml** → **Add 0.5 g in 5 ml (water)**

Aprotinin: **2250 KIU/ml** → **0.6ml X ?**
Fibrinogen: **10 mg/ml** → **1.8 ml X 25 (Aliquots)**
Thrombin: **300 NIH/ml** → **0.2 ml X 25 (Aliquots)**
CaCl2: **0.000680180 mol/ml** → **0.2 ml X 25 (Aliquots)**

For a Final Volume of 0.6 ml (Per Well) Add:

Aprotinin: **135 KIU** → **0.06 ml**
Fibrinogen: **1.8 mg** → **0.18 ml**
Thrombin: **0.75 NIH Units** → **0.005 ml**
CaCl2: **11.6 µmol** → **0.017 ml**

Stock Solution Preparation

Aprotinin

1. Take out of freezer and thaw.
2. Place 0.6 ml in each tube

Fibrinogen

6. Warm 45 mL PBS to 37°C.
7. Add fibrinogen (slowly sprinkle on top)
   (gently agitate, do not vortex or stir)
8. Filter through a 0.20 μm syringe filter
9. Aliquot
10. Store at –80°C.

Thrombin

5. Dissolve thrombin powder in PBS
6. Filter through a 0.20-um syringe filter
7. Aliquot
8. Store at –80°C.

$CaCl_2$

1. Dissolve $CaCl_2$ in distilled water
2. Filter through a 0.20-um syringe filter
3. Aliquot
4. Store at –80°C

Analysis Protocols

Making collagen gel for embedding EBs

1) Prepare one solution of 1N NaOH and 1mL of 10x PBS. Keep them on ice.
2) Put the amount collagen needed and pour it in an ependorf tube. Do it in the hood with sterile forceps. Keep the collagen on ice
3) Here are the proportion for the different components of the gel:

- 9 parts collagen in 1 part 10x PBS
- 23 micro liters of 1N NaOH in 1 mL of collagenase

For example:

- 500μL of collagenase
- 55 μL of PBS
- 11.5 μL of NaOH

4) First, mix PBS and NaOH. Then, add collagen. Keep the gel on ice.
5) Take the plate with the EBs
6) Pour the solution in a falcon tube
7) You can either spin down the tube at 700tr/min for 2 minutes or let the EBs sink. The last solution is better.
8) Suck the medium and wash the cells with PBS
9) Same step as 7)
10) Make a small mold and put it in a small petri dish
11) Take the gel and pour it in the falcon tube where the cells are
12) Take the whole gel with the EBs and pour them in the mold.
13) Leave the mold in the petri dish in the fridge for about 15 minutes so the EBs sink in the gel
14) Then, put the petri dish in the incubator for about 30 minutes
15) After 30 minutes in the incubator, remove the mold
16) Add about 7mL of 3.2% PFA to fix the cells
17) Leave the dish in the fridge for 1 to 2 hours
18) Remove the PFA and add PBS for 1 hour
19) Remove the PBS and add 70%EtOH overnight
20) Now, we can embed the sample from step 4 of the embedding protocol (protocol 9).

Sample Preparation for Cryosectioning

1. Fix sample in formalin for 24 hours.
2. Store sample in DI-H_2O.
3. Exchange DI-H_2O for a 30% sucrose solution (some uses graded solution for a few hours each). I usually leave my samples in sucrose for ~12 hours.
4. Soak sample in HistoPrep to allow for embedding media infiltration (may cause sample shrinkage).
5. Mount sample in HistoPrep and allow it to freeze for at least ~15min in the cryostat.

NOTES:
There really is no set protocol for cryosectioning. Some trial and error may be necessary before an optimal system is found for your samples.

Cryosectioning technique

Slide-Pre-Treatment

1. Place slides in slide rack
2. Place into chromic-sulphuric acid (glass cleaner) for 2 hours
3. Place under running tap water until water is completely clear
4. Let slides dry

Gelatin Coating

1. Add 5g of gelatin to 500 ml of heated dH2O (60^{o}C)
2. Add 0.425g of chromium alume and stir
3. Let cool down slowly to room temperature
4. Dip slides slowly 3X
5. Let dry in 37^{o}C incubator overnight

Cryosectioning Scaffolds

16. Rinse scaffolds in ddH2O (3X 15 min) and place into wells of a 12 well culture plate.
17. Completely cover scaffolds with tissue freezing media (TFM) (Fisher Scientific) and place under a vacuum at 635 mm of Hg for 4h
18. Cryosectioned at –30oC (Ames Lab-Tek cryostat, Elkhart, IN).
    a. I found 30 μm sections worked best
19. Maintain sections at 40 oC overnight on a slide warmer.
20. Prior to staining, immerse slides in water for 10 minutes to remove the TFM.
    a. you may need to play around with this step as it is necessary to remove all TFM but you also do not want your sections to detach from your slides.
    b. I found that dipping individual slides into and out of the water after being submersed for 10-15 min in the water worked best. This way you can keep an eye on your sections to determine how much punishment they can handle ☺.
21. Stain sections with either Gomori's trichrome or toluidine blue.

Paraffin Embedding

Materials

80% EtOH
95% EtOH
100% EtOH
Hemo-De
Paraffin Wax
Omni tissue cassettes
Forceps

Preparation

Day 1: Fill a container with paraffin pellets and place in warm water bath, 58 ° C, will take approximately 2-4 hrs for wax to melt

Day 2: Turn on Leica EG1160 Paraffin Embedding Center (refer to Leica manual located next to machine) and make sure paraffin reservoir is filled with paraffin pellets. The machine needs approximately 4 hrs to warm-up prior to operation.

Method

Sample Dehydration (Day 1)

1. Place cell/polymer construct in tissue cassette.
2. Place cassette in 80% EtOH for 1 hour.
3. Place in 95% EtOH for 1 hour. Repeat.
4. Place in 100% EtOH for 1 hour. Repeat.
5. Place in 50% by volume 100% EtOH / 50% Hemo-De for 1 hour. Repeat.
6. Place in 100% Hemo-De for 1 hour. Repeat.
7. Place in paraffin wax at 58°C for one hour.
8. Transfer to more paraffin wax at 58°C for 24 hrs.

Embedding Sample (Day 2)

1. Remove cassettes from paraffin and place in cassette bath of the Leica PEC
2. Using forceps remove cassettes from bath, open, and remove sample
3. Place a metal mold under paraffin dispenser and add a small amount of wax to mold using foot peddle to dispense
4. Position sample as desired in wax, and cover with more paraffin
5. Place mold on cold plate, once fully cooled remove embedded sample from mold. Place samples in freezer until ready to section.
6. Section with microtome at 5-10 um.
7. Place “tapes” of sections on colorfrost slide with 30% EtOH, and place slide on slide warmer (60°C) for 1 min. Remove and leave out to dry at room temperature overnight.
8. Before staining place slide(s) on slide warmer for 45 minutes at 60°C.
9. Stain as desired.

Notes:

- Hemo-De is also known as Citrisolv, and is purchased from Fisher (22-143-975)
- Paraffin wax is Paramat Extra from Electron Microscopy Sciences
- Use pencil to mark cassettes as marker will wash off in Citrisolv
- Dehydration steps can be broken up into two days by leaving the cassettes in the second 100% EtOH bath overnight

De-paraffinizing for staining

Materials:

Xylene
Ethanol series (100%, 95%, 85%, 70%, 50%, 30%)
PBS
4% Formaldehyde
0.1M Triethanolamine
Acetic anhydride

Procedure:

1) Dewax slides in xylene for 10 min
2) Remove xylene with 100% ethanol for 2 min
3) Rehydrate by putting slides quickly through 100%, 95%, 85%, 70%, 50%, then 30% ethanol
4) Remove ethanol
5) Rinse in PBS for 5 min
6) Post-fix sample in 4% formaldehyde diluted in PBS for 20 min
7) Wash in PBS twice for 5 min
8) To 250 mL 0.1M triethanolamine add 0.625 mL acetic anhydride, mix well on stirrer
9) Dip slides in water and then immerse in acetic anhydride solution for 10 min
10) Wash with PBS twice for 5 min

Staining

Actin Staining

1. Fix cells in 70% acetone/30% methanol for 5 minutes (~1ml per well)*
2. Aspirate fixant, then permeabilize/block with (~1ml per well) 15 minutes:
   a. 3% BSA
   b. 0.1% Tween-20
3. Rinse 3X with PBS for 5 minutes each
4. Incubate coverslip upside down onto 1 μg/ml fluorescently labeled phalloidin for 30 min (use about 50μL of antibody solution per coverslip)
5. Rinse 3X with PBS for 5min. each (~1mL per well)
6. Add 1μg/mL DAPI (in methanol) for 5 min. (~1mL per well)
7. Rinse briefly with ddH_2O
8. Dry in coldroom for ~15 minutes to get rid of excess moisture
9. Coverslips: mount on slides (upside-down) onto Gel-mount w/DABCO
   Hydrogels: store in water in fridge (cover and wrap in parafilm)
10. visualize

* Leave coverslips right-side up in well plates except in the antibody incubations

Antibody Staining (General)

11. fix in 70% acetone/30% methanol for 5 minutes (~1ml per well)*

12. aspirate fixant, then permeabilize/block with (~1ml per well) 15 minutes:
    a. 3% BSA
    b. 0.1% Tween-20

13. rinse 3X with PBS for 5 minutes each

14. incubate coverslip upside down onto 1° antibody for 1 hour in humidity chamber (use about 50μL of antibody solution per coverslip)

15. rinse 3X with PBS for 5min. each (~1mL per well)

16. incubate coverslip as in #4 with 2° antibody (1 hour)

17. rinse 3X with PBS (~1mL) for 5 min. each

18. add 1μg/mL DAPI (in methanol) for 5 min. (~1mL per well)

19. rinse briefly with ddH_2O

20. dry in coldroom for ~15 minutes to get rid of excess moisture

21. Coverslips: mount on slides (upside-down) onto Gel-mount w/DABCO
    Hydrogels: store in water in fridge (cover and wrap in parafilm)

22. visualize

* Leave coverslips right-side up in well plates except in the antibody incubations

LIVE/DEAD Assay (Molecular Probes)

1) Prepare Dye Solution
    a. For 1 ml in PBS (or any other phenol red free cell compatible solution) add:
        i. 0.5 µl Calcein
        ii. 2.0 µl Ethidium Homodimer
2) Add enough dye solution to completely immerse construct or cells
3) Incubate (37°C) for 10 minutes
4) Rinse sample with PBS to remove excess dye
5) Mount cells with Fluromount mounting solution
6) View sample with fluorescence – View live cells under blue excitation (green emission), dead cells under green excitation (red emission)

CFSE Staining

1. Pellet Cells
2. Using 10x CFSE solution, add 1mL 1X CFSE (0.9 mL PBS and 0.1 mL 10X CFSE)
3. Incubate at 37 deg C for 15 min
4. Add a few mLs of PBS
5. Pellet
6. Resuspend in desired volume of media

MSB (Martius Scarlet Blue)(after Lendrum[17])

http://www.histosearch.com/histonet/Jun03/MartiusscarletbluestainfoA.html
Three dyes of different molecular sizes are used in this method to selectively stain connective tissues. A yellow dye, of small molecular size, in the presence of alcoholic phosphotungstic acid, selectively stains red blood cells and sometimes early deposits of fibrin. A red, intermediate sized dye molecule is then used to selectively stain muscle and mature fibrin. Staining of collagen is prevented by treatment of the section with aqueous phosphotungstic acid which removes red staining from collagen after staining in the red dye. A large molecular size blue dye is then used to stain collagen and old fibrin.

SECTION PREPARATION:
Paraffin sections are cut at 3 to 5 mm from tissue fixed in neutral buffered formalin. A control section from foetal lung in hyaline membrane disease, should be included.

REAGENTS REQUIRED:

Martius yellow solution
Martius yellow (CI 10315) 0.5 g
Absolute ethyl alcohol 95 ml
Phosphotungstic acid 2 g
Distilled water 5 ml
Dissolve the dye in absolute ethanol and the phosphotungstic acid in distilled water. Combine the two solutions.

Crystal ponceau solution
Crystal ponceau 6R (CI 16250) 1 g
Distilled water 97.5 ml
Glacial acetic acid 2.5 ml

Aniline blue solution
Aniline blue (CI 42755) 0.5 g
Distilled water 99 ml
Glacial acetic acid 1 ml

1% acetic acid
Glacial acetic acid 1 ml
Distilled water 99 ml

1% aqueous phosphotungstic acid
Phosphotungstic acid 1 g
Distilled water 100 ml

Weigert's haematoxylin

METHOD:

1. Dewax and rehydrate sections.
2. Stain with Weigert's haematoxylin for 5 minutes.
3. Wash in tap water.
4. Differentiate, if necessary, in acid alcohol then blue in running tap water (or suitable alternative) for 5 minutes.
5. Rinse in 95% ethanol.
6. Stain with martius yellow for 3 minutes.
7. Rinse in distilled water.
8. Stain in crystal ponceau 2R solution for 10 minutes.
9. Drain the stain from the slide.
10. Mordant and differentiate with 1% phosphotungstic acid for 5 minutes.
11. Rinse in distilled water.
12. Stain in aniline blue for 1 minute.
13. Rinse briefly in 1% acetic acid.
14. Dehydrate, clear and mount with a neutral mounting medium.

General Antibody staining using an HRP-conjugated secondary

Materials

- 10% formalin
- PBS
- FBS or BSA
- 3% H_2O_2
- 0.1% Triton-X in PBS
- primary antibody
- secondary antibody (HRP-conjugated)
- AEC (or other suitable HRP chromogen)
- TBS (0.05 M Tris-HCl, 0.15 mM NaCl, pH 7.6)
- 37 mM NH_4OH

Methods

1. Remove media and rinse cells with PBS
2. Incubate cells with 10% formalin at room temp for 10 minutes
3. Rinse 2x with PBS
4. Permeabilize fixed cells by incubating in Triton-X solution for 2 minutes at room temp
5. Rinse 4x with PBS over 5 minutes
6. Remove PBS and incubate cells in H_2O_2 for 5 minutes (this quenches endogenous peroxidase activity of the cells)
7. Rinse with diH_2O, then incubate in TBS for 5 minutes
8. Add 1° antibody (i.e. anti-PCNA), leaving 2 wells untreated. Incubate for 60 minutes at room temp in a humidified chamber
9. Rinse 3x with PBS over 5 minutes
10. Add 2° antibody (i.e. anti-mouse IgG HRP), leaving 1 previously untreated well untreated. Incubate for 40 minutes at room temp in a humidified chamber
11. Rinse 3x with PBS over 5 minutes
12. Incubate all wells with AEC solution for 5-15 minutes
13. Rinse with diH_2O
14. Counterstain with Mayer's hematoxylin
    a. Cover cells with hematoxylin and incubate for 5 minutes
    b. Rinse with diH_2O
    c. Add NH_4OH sol'n and incubate for 1 minute. Remove and repeat until stain turns blue.
    d. Rinse with diH_2O
15. Mount with aqueous mounting medium

Notes:

- Make all antibody solutions in 3% BSA or FBS in PBS (blocking buffer).
- This protocol allows for two staining controls: a) one well that receives no 1°, but has 2° and AEC, and b) one well that receives neither 1° nor 2°, but does have

AEC.

- Staining control wells should be kept in blocking buffer when other wells contain antibody solutions.
- Antibody dilutions are not given here, as they will vary with the antibody source.
- Aqueous mounting media cannot be used with some chromogen solutions.

Staining ES cells for SSEA-1 – Method 1: 6-Well Plate

1. Coat wells in 6-well dish with gelatin.
2. Plate cells onto each well. Allow cells to adhere overnight.
3. Once the cells have adhered, suck off the media, being careful not to disturb the adhered cells.
4. Add a few drops of 4% paraformaldehyde to fix the cells.
5. Incubate in a humidified chamber 30-60 minutes (ie wet paper towels).
6. Prepare 1X PBS. (Add 200 ml 10X PBS to 1800 ml distilled water).
7. Suck off remaining paraformaldehyde.
8. Wash wells with 1X PBS twice.
9. Dilute 1˚ antibody (SSEA-1, or MC-480) 1:10 in DAKO antibody diluent or in 1% BSA in PBS.
10. Add enough 1˚ antibody to coat surface.
11. Incubate in a humidified chamber 30-60 minutes.
12. Suck off remaining 1˚ antibody.
13. Wash in 1X PBS twice.
14. Dilute 2˚ antibody (anti-mouse IgM) 2:1000 in DAKO antibody diluent or in 1% BSA in PBS.
15. Add a few stops of 2˚ antibody to coat slide surface.
16. Incubate in a humidified chamber 30-60 minutes.
17. Suck off remaining 2˚ antibody.
18. Wash in 1X PBS.
19. Wash in distilled water.
20. Use Fluoromount-G to mount coverslip.
21. Visualize under fluorescence microscope

Staining ES cells for SSEA-1 – Method 2: Glass Slides

1. Coat center of slides with gelatin.
2. Plate cells onto each slide. Allow cells to adhere overnight.
3. Once the cells have adhered, carefully remove excess media with Kim wipe.
4. Add a few drops of 4% paraformaldehyde to fix the cells.
5. Incubate slides in a humidified chamber 30-60 minutes (ie wet paper towels).
6. Prepare 1X PBS. (Add 200 ml 10X PBS to 1800 ml distilled water).
7. Remove remaining paraformaldehyde.
8. Wash slides with 1X PBS twice.
9. Dilute 1˚ antibody (SSEA-1, or MC-480) 1:10 in DAKO antibody diluent or in 1% BSA in PBS.
10. Add a few stops of 1˚ antibody to coat slide surface.
11. Incubate slides in a humidified chamber 30-60 minutes.
12. Remove remaining 1˚ antibody.
13. Wash in 1X PBS twice.
14. Dilute 2˚ antibody (anti-mouse IgM) 2:1000 in DAKO antibody diluent or in 1% BSA in PBS.
15. Add a few stops of 2˚ antibody to coat slide surface.
16. Incubate slides in a humidified chamber 30-60 minutes.
17. Remove remaining 2˚ antibody.
18. Wash in 1X PBS.
19. Dehydrate slides in 70% ethanol (3 min), 95% ethanol (3 min), 100% ethanol (3 min), xylene (at least 5 min).
20. Mount slides by placing several drops of cytoseal onto cells. Apply coverslips and remove bubbles by pressing on coverslips.
21. Visualize under fluorescence microscope

Immunofluorescent Staining (and DAPI)

Materials

- 4% formaldehyde
- PBS
- Blocking buffer (0.1% Tween-20 in PBS)
- 0.1% Triton-X in PBS
- primary antibody
- secondary antibody (fluorescent conjugate)
- DAPI: diluted 1:100 or 1 ug/ml in PBS
- Fluromount mounting solution

Methods

1. (Optional) Tether cells to coverslip
2. Remove media and rinse cells with PBS
3. Incubate cells with 4% formaldehyde at room temp for 10 minutes
4. Rinse 2x with PBS
5. Permeabilize fixed cells by incubating in 0.1% Triton-X solution for 2 minutes at room temp
6. Rinse 4x with PBS over 5 minutes
7. Add 1° antibody to wells or invert coverslip (cell side down) over large drop of 1° antibody on Parafilm..
8. Incubate for 60 minutes at room temp in large slide box with moist Kimwipe.
9. Rinse 3x with PBS over 5 minutes
10. Add 2° antibody to all wells or invert coverslip (cell side down) over large drop of 2° antibody on Parafilm.
11. Incubate for 30 minutes at room temp inlarge slide box with moist Kimwipe
12. Rinse 3x with PBS over 5 minutes
13. Counterstain with DAPI diluted 1:100 (or 1μg/mL in PBS)
14. Incubate for 1-5 minutes
15. Rinse several times with PBS
16. Rinse 1x with DI H_2O
17. Mount with Fluromount mounting solution

Notes:

- Make all antibody solutions in blocking buffer
- Staining control wells should be kept in blocking buffer when other wells contain antibody solutions.
- Antibody dilutions are not given here, as they will vary with the antibody source.

Suggested Antibody and Stain Dilutions

Stains

DAPI (from working stock)	1:100
Fluorescent Phalloidin	1:100

Antibodies

1˚	SSEA	1:20
2˚	IgM-PE	1:500
1˚	Troma-1	1:10
2˚	Texas red IgG donkey-α-rat	1:100
	IgG-PE goat-α-rat	1:100
	IgG_{2a}-PE goat-α-mouse	1:100

Primary Antibodies

Primary Antibody	Antigen species	Type	Antibody species	Company	Catalog #	Dilution
~~o AFP~~	~~Human~~	~~Monoclonal~~	~~Mouse IgG1/IgG2a~~	~~RDI~~	~~RDI-TRK3F16-5H7 and 4A3~~	
~~o AFP~~	~~Human~~	~~Polyclonal~~	~~Goat~~	~~Dako~~	~~A0008~~	~~1:2500~~
o AFP	Human	Polyclonal	Rabbit	Dako	A0008	1:2500
o Albumin	Human	Polyclonal	Rabbit	Dako	A0001	1:500
o Cytokeratin 7				Dako	M7018	1:25
~~o Cytokeratin~~	~~Human~~	~~Polyclonal~~	~~Rabbit~~	~~Dako~~	~~A0575~~	~~1:500~~
~~o Cytokeratin 8~~	~~Human~~	~~Monoclonal~~	~~Mouse IgM, kappa~~	~~Dako~~	~~M0631~~	
o Cytokerain (PAN) **	Human	Monoclonal	Mouse IgG1	RDI	RDI-PR61031	
o Nestin	Rat	Monoclonal	Mouse IgG1	BD	611658	1:500
o Insulin	Porcine	Polyclonal	Guinea Pig	Dako	A0564	1:100
o Insulin **	Human	Monoclonal	Mouse IgG1	Sigma	I2018	1:1000
~~o Glucagon~~	~~Porcine~~	~~Monoclonal~~	~~Mouse IgG1~~	~~Sigma~~	~~G2654~~	~~1:2000~~
o Glucagon **	Human	Polyclonal	Rabbit	Chemicon	AB932	1:250
~~o Somatostatin-14~~	~~Human~~	~~Polyclonal~~	~~Rabbit~~	~~Chemicon~~	~~AB1976~~	~~1:1600~~
o Somatostatin *	Human	Polyclonal	Rabbit	Dako	A0566	1:500
o Pancreatic polypeptide (PP) *	Human	Polyclonal	Rabbit	Biogenex	AR066	1:500
o FoxA2 / HNF-3β	Mouse	Monoclonal	Mouse IgG1	Melton		
o Pax-6	Mouse	Monoclonal	Mouse IgG1	Melton		
o Shh	Mouse	Monoclonal	Mouse IgG1	Melton		
o PDX-1 #	Mouse	Monoclonal	Mouse IgG1	Melton		

Monoclonal	Polyclonal
Insulin	Insulin
CK PAN	AFP
Nestin	Albumin
PDX-1	Somatostatin
Pax6	PP
FoxA2 / HNF-3β	Glucagon
Shh	

Different combinations:

1) DAPI / CK / Insulin
2) DAPI / CK / AFP
3) DAPI / CK / Abumin
4) DAPI / Insulin / Glucagon
5) DAPI / Insulin/ PP
6) DAPI / Insulin / Somatostatin
7) DAPI / Nestin / Insulin
8) DAPI / Nestin / Albumin
9) DAPI / Nestin / Glucagon
10) DAPI / Nestin / AFP
11) DAPI / Insulin / PDX
12) DAPI / Insulin / Shh or FoxA2 or Pax6

- Crossed out antibodies are not required for the experiments
- ** indicates must be bought * indicates may be needed for secondary experiments
- # obtain from Melton

Secondary Antibody	Antigen species	Antibody species	Company	Catalog #	Dilution
o FITC	Rat	Anti-mouse IgG1	PharMingen	553443	1:500
o FITC		Anti-rabbit	Biogenex		1:200
o FITC		Anti-guinea pig	Biogenex		1:500
o Texas Red	Rat	Anti-mouse IgG1	Biogenex		1:200

Control	Antigen species	Antibody species	Company	Catalog #	Dilution
o Rabbit IgG					
o Goat IgG					
o Mouse IgG1					

Binding Strength of Serum Proteins to Hyaluronic Acid Gels

1. Swell gels overnight in PBS to bring to equilibrium swelling.
2. Incubate gels for 45 minutes at 37°C in 15% solution of FBS in PBS.
3. Aspirate off FBS solution.
4. Rinse three times with 500μL of DI-H_2O per gel. Allow each wash to sit on gels for 20 minutes at room temperature. Save supernatants from each rinse step in microcentrifuge tubes.
5. Rinse once with 10% isopropanol. Allow isopropanol to sit on gels for 20 minutes. Save supernatants in microcentrifuge tubes.
6. Rinse once with 30% isopropanol. Allow isopropanol to sit on gels for 20 minutes. Save supernatants in microcentrifuge tubes.
7. Rinse once with 50% isopropanol. Allow isopropanol to sit on gels for 20 minutes. Save supernatants in microcentrifuge tubes.
8. Rinse once with 70% isopropanol. Allow isopropanol to sit on gels for 20 minutes. Save supernatants in microcentrifuge tubes.
9. Place open microcentrifuge tubes in hood overnight to allow isopropanol to evaporate.
10. Freeze dry supernatants.

SDS-PAGE

1. Resuspend supernatants in 30μL SDS-PAGE sample buffer.
2. Heat samples in boiling water bath for 4 minutes.
3. Remove Ready Gel from storage pouch.
4. Remove comb from gel and rinse with DI-H_2O.
5. Cut along dotted line at the bottom of Ready Gel Cassette with a razor blade.
6. Pull the clear tape at the bottom of the Ready Gel Cassette to expose the bottom edge of the gel.
7. Repeat for second Ready Gel. (NOTE: use mini cell buffer dam if only one gel is to be run.)
8. Place Gel Cassette Sandwich into electrode assembly with short plate facing inward.
9. Slide electrode assembly into clamping frame.
10. Press down on the electrode assembly while closing the 2 cam levers of the clamping frame.
11. Lower the inner chamber into the mini tank. Fill the inner chamber with ~125mL of 1X SDS running buffer until the level reaches halfway between the tops of the taller and shorter glass plates of the gel cassettes. (NOTE: Do not overfill inner chamber.)
12. Add ~200mL of 1X SDS running buffer to the Mini Tank.
13. Load 10μL of sample into each well. Load samples slowly to allow them to settle evenly on the bottom of the wells. Remember to use special gel loading tips. Record what samples are in each lane. Always put MW marker in Lane 1 for orientation purposes. (NOTE: SIGMAMarker already contains sample buffer)
14. Place lid on mini tank.

15. Insert electrical leads into power supply.
16. Turn on power and apply 150 volts. Run time is 25-35 minutes. Keep an eye on dye marker bands.
17. When dye reaches the bottom of the gel, turn off power supply and disconnect the electrical leads.
18. Remove tank lid and carefully lift out inner chamber assembly. Pour off running buffer. (NOTE: Running buffer can be re-used.)
19. Remove gel cassette sandwiches from assembly.
20. To remove the gel from a Ready gel cassette, slice the tape along the sides of the Ready Gel Cassette where the inner glass plate meets the outer plastic plate.
21. The green plastic Gel Releaser may be used to pry the plates apart. Remove the gel from the glass plate by floating it off in fixative solution. Agitate gently until the gel separates from the plate.
22. Stain the gel using Silver Stain Plus protocol. The fixative step is an acceptable stopping point if there is not enough time to finish the staining procedure.

Microarray Protocols

1. Isolating RNA from the samples to be compared
2. Converting the RNA samples to labeled cDNA via reverse transcription; this step may be combined with aRNA amplification
3. Hybridizing the labeled cDNA to identical membrane or glass slide arrays
4. Removing the unhybridized cDNA
5. Detecting and quantitating the hybridized cDNA, and
6. Comparing the quantitative data from the various samples

**This doesn't include array fabrication

I. RNA Isolation

1. Aspirate media from cells and wash once with Phosphate Buffered Saline (PBS).
2. Add 5mL Trypsin (1X) and incubate at 37°C for approximately 5 minutes.
3. Visually inspect plate to ensure cells have detached.
4. Add 4mL of cell media to plate and pipet cell suspension into a 50mL polypropylene conical-bottom tube.
5. Wash plate with an additional 4mL of media and add it to the tube.
6. Pellet the cells by centrifugation at 1000 rmp for 6 minutes at 4 °C and discard supernatant.
7. Add 2mL Trizol (Life Technologies; Cat # 15596-014) per ~$2X10^6$ cells to the pellet and pass the suspension through an 18 gauge syringe several times to disrupt the pellet.
8. Incubate the sample at room temperature for 5 minutes.
9. Add 0.2mL chloroform per 1mL Trizol and shake vigorously for 1 minute.
10. Incubate at room temperature for 2.5 minutes.
11. Remove cellular debris by centrifugation at 4000 rpm for 15 minutes at 4 °C.
12. Transfer supernatant to 1.2mL microcentrifuge tubes (0.5mL/tube) and an equal volume of isopropanol to precipitate the RNA.
13. Incubate at room temperature for 15 minutes.
14. Centrifuge at 15,000 rpm for 15 minutes to pellet RNA.
15. Discard supernatant and resuspend the pellet in 70% ethanol. RNA can be stored in 70% ethanol at -20°C until use.
16. Prior to use, centrifuge at 15,000 rpm for 15 minutes and discard supernatant.
17. Resuspend pellet in diethylpyrocarbonate (DEPC) treated water or Rnase-free TE buffer for labeling.

II. cDNA Synthesis and Labeling

1. Prepare a labeling reaction master mix containing 500μM dCTP, 500μM dATP, 500μM dGTP, 100μM dTTP, 1mM dithiothreitol (DTT) and 1X RT buffer.

2. To 10μg of total RNA in a microcentrifuge tube, add 2μg of oligo(dT) and DEPC-treated water to a total volume of 10μL.
3. Incubate the reaction mixture at 70°C for 10 minutes and chill on ice for one minute.
4. Add 15μL of RT labeling mix, 3μL cyanine3-dUTP or cyanine5-dUTP (1mM), 2μL Superscript II RT (200U/μL; Life Technologies; Cat# 18064-014).
5. Mix thoroughly and incubate at 42°C for 2 hours.
6. Briefly centrifuge the reaction and add 1.5μL of 20mM EDTA to stop the reaction.
7. Add 1.5μL of 500mM NaOH and heat at 70°C for 10 minutes to degrade the RNA.
8. Neutralize reaction by adding 1.5μL of 500mM HCl.
9. Unincorporated fluorescent nucleotides are removed by glass fiber filtration using GFX columns (Pharmacia Cat# 27-9602-01) and the instructions provided by the manufacturer.
10. Elute purified products using 50μL of TE pH 8.0 and dry the probe to completion.
11. Resuspend the probe in 10μL of DEPC treated water.
12. Repeat steps 2-11 for other probe.

III. Prehybridization

1. Prepare prehybridization buffer containing 5X SSC, 0.1% SDS, and 1% bovine serum albumin (BSA)
2. Prepare 2X hybridization buffer containing 50% formamide, 10X SSC, and 0.2% SDS.
3. Place slides to be analyzed into a Coplin jar, fill with prehybridization buffer, and incubate at 42°C for 45 minutes.
4. Wash the slides by dipping 5 times in room temperature MilliQ water.
5. Dip slides in room temperature isopropanol and air dry.

*Slides should be used immediately following prehybridization.

IV. Hybridization

1. Combine 10μL each of purified Cy3- and Cy5-labeled probes, mix well and add
   1μL COT1-DNA (20μg/μL; Life Technologies; Cat# 25279-011) and 1μL Poly(A)-DNA (20μg/μL; Pharmacia; Cat# 27-7836-01) to block nonspecific hybridization.
2. Heat the probe mixture at 95°C for 3 minutes to denature.
3. Centrifuge the probe at maximum angular velocity for 1 minute.
4. Combine the probe with an equal volume of 2X hybridization buffer that has been heated to 42°C.

5. Apply the labeled probe to a prehybridized microarray slide and cover with a 22mm X 60mm polyethylene hydrophobic coverslip.
6. Place the slide in a sealed hybridization chamber, add 20μL of water to the chamber at the end of the slide.
7. Place the sealed chamber in a 42°C water bath and incubate for 16-20 hours.
8. Remove the array from the hybridization chamber, taking care not to disturb the coverslip.
9. Place the slide in a staining dish containing low-stringency wash buffer containing 1X SSC and 0.2% SDS at 42°C.
10. Gently remove the coverslip while the slide is in solution and agitate for 4 minutes.
11. Wash the slide at high stringency in a staining dish containing 0.1X SSC and 0.2% SDS at room temperature, agitating for 4 minutes.
12. Wash the slide in 0.1X SSC, agitating for 4 minutes.
13. Allow slides to air dry.

MTT Cell Assay

Plate cells in 96 well plate at $3x10^3$ to $1x10^5$ cells/well. Seeding density depends on purpose and cell line used. For growth curves, cells should be seeded at approx. $3x10^3$ cells/well. For binding or toxicity assay, cells should be seeded at $3x10^4$ to $1x10^5$ cells/well.

Seeding densities can also be based on TC area and split ratio. For growth curves split cells from near confluence at approx. 1:32 to 1:16 (if cells can stand this). For binding and toxicity, seed cells from near confluence approx. at 1:4 to 1:2. These splits should be adjusted if harvested flask is not near confluence or is overconfluent (avoid using overconfluent cells). For calculations the plating area per 96 well plate (center wells) is 60 wells x 0.33 cm^2/well = 20 cm^3. Mix cell suspension so that cells harvested from 10 cm^2 are suspended in each 6 ml of media for a 1:2 split. Cells should be counted to determine and record seeding density.

split	harvest T-150 in	dilute at	seed
1:2	15 ml	1:6	100 □l/well
1:4	"	1:12	"
1:8	"	1:24	"
1:16	"	1:48	"
1:32	"	1:96	"

To plate cells, harvest flask of cells, transfer to sterile tube, and dilute in plating media to approx. 10^6 cells/ml (estimated). Mix well and remove aliquot for counting (place tube of cells on ice while counting aliquot).

Count with hemocytometer of Coulter counter.

Note: For cell growth curves, flasks of cells should be harvested at subconfluence. For special purposes cells may be serum starved or GLN starved for 18 to 24 hours prior to harvesting for plating. Cells at high density should never be used because clumps of cells will result.

Prepare 96 well plates to be seeded: open packages, label, pre-dose, etc. For example, for dose response curves, drugs can be pre-diluted in plates leaving 100□l/ well of 2X drug concentrations. Addition of 100 □l/well of cell solution will result in final 1X drug concentration.

Dilute cells to $1x10^4$ to $3x10^5$ cells/ml in plating media. Mix >6 ml of dilution per plate to be seeded. Mix well and transfer diluted media to sterile multipipetter trough.

Load multipipetter with 6 or 10 tips depending on seeding strategy: by column (6) or by row (10). Plate seeding can affect results if one barrel of multipipetter consistently

delivers more or less volume of cell suspension. In general one should seed by rows if test groupings will be by columns.

Using multipipetter, carefully pipette 100 □l/well of diluted cell suspension into CENTER 60 WELLS of 96 well plate (10x6 matrix). When drawing up cell suspension, expel once to mix suspension in trough. After drawing up 100 □l/tip, visually confirm that liquid levels are equal in each tip.

If drugs, etc. were not pre-added to wells, add 100 ml/well of media containing 2X concentration of substance to each column of wells. This can be done immediately or after the cells have adhered overnight. Waiting will avoid drug effects on seeding efficiency. For growth curves, add drugs on same day cells are seeded.

Fill exterior wells with 200 to 250 □l/well of sterile H2O or PBS using repeater pipette. This will reduce evaporation from inner wells.

Incubate plates.

Analyze plates at various times after seeding according to purpose. For growth curves, times will depend on growth rate of cells. For fast growing cells, plates should be analyzed at 1, 2, 3, 4, 5 and 7 days. For intermediate and slow growing cells, plates should be analyzed at 1, 3, 5, 6, 10, and 14 days with a media change at day 7.

MTT ASSAY: to determine relative cell density.

Prepare MTT solutions.
10X MTT: Completely dissolve 1 tablet phosphate buffered saline pH 7.4 in 200 ml MQ water. In a 200 ml beaker, add 100 ml of this to 0.5 g MTT. Cover and stir 4 -24 hours at 4^{o}C. DO NOT HEAT. Filter through 0.2mm filter into a sterile 100 ml bottle. Store at 4^{o}C.
HCl in Isopropanol: Add 1 ml 12 N HCl to 299 ml isopropanol in a 500 ml bottle. Store at 4^{o}C.

Dilute 1 part 10X MTT solution with 9 parts serum free media (media without FBS is okay). May want to use media without phenol red.

Remove media from wells by dumping off and blotting upon paper towels. Plates must be shaken hard when dumping due to surface tension in wells. Give the plates several hits on the towels to ensure all media is removed. Check through microscope to ensure cells were not lost during media removal.

Fill all wells with 100 µl/well serum-free media plus MTT.

Incubate plate for 2 to 4 hours (4 hrs probably better). Must be consistent if comparing results from different plates.

Add 100 µl/well of acidic isopropanol solution.

Aggressively triturate solution in wells using micropipetter, to detach cells and break up purple crystals. Avoid bubbles and spillage.

Place plate on rocker for 30 minutes.

Read optical densities on plate reader using 560 nm filter with 650 nm reference filter. OD560-650 should be proportional to cell number. Columns or rows of outer wells originally filled with PBS should be blank.

RNA Quantification

1. Pour off ethanol from RNA sample carefully – let air dry 5 min.
2. Add 100 µl 0.1% DEPC water, put samples in oven (55-60 ºC) for 10 min.
3. Blank UV-vis (260 nm) with 0.1% DEPC water
4. Prepare samples
    a. 990 µl of 0.1% DEPC water
    b. add 10 µl sample
    c. mix thoroughly
5. To find volume to add (V_{add}) to get 10 ng/µl:
    a. (OD_{260nm}*4000*90 (resuspended concentration minus sample - V_s))/10 (wanted concentration) = V
    b. $V-V_s = V_{add}$

Fixing Cells

Formaldehyde/Formalin Fixation

Cover cells with or submerse constructs in 10% formalin (4% formaldehyde) for not longer than 24 hours.

Remove formalin or remove from formalin and store in dH2O until staining or dehydrating

10% formalin can be found in the histology lab

Paraformaldehyde - Triton X-100 Fixation

1. Briefly rinse cells with PBS at 37°C.

2. Fix samples with 4 % paraformaldehyde in PBS for 10 minutes at RT.

3. Wash samples 3x with PBS, for two minutes each time.

4. Permeabilize cells with 0.2% Triton X-100 diluted with PBS, for 5 minutes.

5. Rinse samples 4x with PBS, for two minutes each.

Cell monolayers

Paraformaldehyde Fixation

1. Prepare a fresh 4% solution of paraformaldehyde by heating in buffer (PBS, TRIS, HEPES, Veronal, etc.) to 55- 60° C. Add several drops of 1N NaOH and stir until dissolved. Allow cooling to room temperature before use.

2. Wash the coverslips once with PBS and then fix with PFA for 15min.

3. Wash the coverslips once with PBS and then permeabilise the cells with 0.2% Triton-X100 in PBS for 5min.

4. Wash the cells once with PBS and then quench in fresh 0.1% sodium borohydride in PBS for 5min.

5. Wash three times in PBS and proceed to staining protocol.

Staining

1. Dilute primary antibody as appropriate in 1% BSA in PBS. Centrifuge the diluted antibody for 5 min at 12,000 x *g* in a refrigerated microcentrifuge prior to use will remove aggregated material and reduce background.

2. Incubate the coverslip with a small volume of diluted primary antibody for 1h.

3. Dilute the secondary fluorescent antibody as appropriate in either PBS or 1% BSA in PBS, depending on the background staining. Again, centrifuging the diluted antibody for 20 min at 12,000 x *g* in a refrigerated microfuge will reduce background.

4. Incubate the coverslip with a small volume of diluted secondary antibody for 45min.

5. Wash coverslips three times with PBS.

Mounting

Mount in appropriate mounting media containing an anti-photobleaching reagent (glycerol, Mowiol, BABB etc. with 0.6% DABCO, p-phenylenediamine, etc). Coverslip, seal with nail polish and store in the dark at 4 degrees C.

DAPI

1. Equilibrate cells in PBS.

2. Dilute DAPI 1:300 to 1:1,000 with PBS.

3. Incubate cells for up to 5 minutes.

4. Wash 3x with PBS.

Hoechst 33258 DNA assay

PBE buffer
100 mM Na_2HPO_4 (7.1 g/500 ml)
10 mM EDTA (1.86 g/500 ml)
pH 6.5

Papain solution
125 ug/ml papain (Worthington Biochemical; 0.1 ml of 25 mg/ml stock per 20 ml)
10 mM cysteine (0.035 g/20 ml)
in PBE buffer

10X TNE solution
12.11 g Tris base
3.72 g EDTA
116.89 g NaCl
in 1 L total diH_2O
pH 7.4
filter through 0.45 um
store at 4°C

Assay solution
10 ul of 1 mg/ml Hoechst 33258 (diluted in 1X TNE)
10 ml 10X TNE
90 ml diH_2O

Hoechst 33258 DNA assay

1. Prepare working calf thymus DNA solution at 10 ug/ml by adding 10 ul of 1 mg/ml DNA stock solution to 990 ul of 1X TNE.
2. Prepare the following standards in individual wells of a black 96 well plate:

ul DNA working solution	ul 1X TNE	resulting ug DNA
0	100	0.0
10	90	0.1
20	80	0.2
30	70	0.3
40	60	0.4
50	50	0.5

3. To other individual wells, add 4 ul of digested sample and 96 ul of 1X TNE.
4. Add 100 ul of assay solution to each sample and standard well.
5. Read each sample on fluorometer (360 nm/ 465 nm) by selecting Hoechst DNA assay on the flourometer and loading your plate.
6. Prepare standard curve and calculate DNA in sample wells, taking dilution into account.

Micro BCA Protein Assay Protocol (Pierce)

Detection range: 0.5-20 μg/ml

Materials
Pierce Micro BCA Protein Assay Kit, Catalog # 23235

Methods

1. Dilute 2 mg/ml BSA stock solution (provided in 1 ml ampules) according to chart below, preferably with same diluent as your sample.
2. Pipet 1 ml of each standard and sample in 15 ml centrifuge tubes. Blank tubes should consist of 1 ml of diluent.
3. Prepare a working solution of BCA Reagents, assuming 1 ml working solution per sample or standard. Add 25 parts Reagent MA to 24 parts Reagent MB and 1 part Reagent MC (25:24:1 Reagent MA:MB:MC) and mix well. The working solution will be a clear-green color and is stable for several days at room temperature in the dark.
4. Add 1 ml of the working solution to each centrifuge tube containing samples/standards.
5. Incubate the tubes at the 60°C for 1 hour.
6. Cool all tubes to room temperature on shaker plate.
7. Measure the absorbance at 562 nm on the UV-Vis. Zero the UV-Vis using diH_2O, and read all samples within 10 minutes. The BCA reaction has no true end point, so color development continues even at room temperature.

Standard dilutions:

Vial	Diluent vol. (ml)	BSA vol. (ml)	Final BSA conc. (μg/ml)
A	4.5	0.5 ml stock	200
B	4	1 ml of vial A	40
C	4	4 ml of vial B	20
D	4	4 ml of vial C	10
E	4	4 ml of vial D	5
F	4	4 ml of vial E	2.5
G	4.8	3.2 ml of vial F	1
H	4	4 ml of vial G	0.5
I	8	0	0

Determining intranuclear Expression by Flow Cytometry

Description

To determine the Oct4 protein expression of ES cells as a measure of their differentiation status.

Reagents

- Mouse anti-mouse Oct3/4 antibody (IgG1 isotype) (Transduction Laborotories)
- Goat anti-mouse IgG (Fc specific) FITC Conjugate (Sigma, F-2772)
- Hank's Buffered Saline Solution with 2% FCS (HF)
- Coulter Permeabilization Kit (Beckman Coulter)

Method

1. Trypsinize cells (10^6) and generate a single cell suspension in 100 mL of HF
2. Fix with reagent 1 (100 mL) of the Coulter Permeabilization kit for 15 min at RT.
3. Wash with 1 ml of HF
4. Permeabilize with 100 mL of reagent 2 from the kit for 5 min at RT.
5. Add 1 mL of the Oct4 Ab and incubate at RT for 20 min.
6. Wash twice with 1 ml of HF
7. Add 1 mL of anti-mouse FITC conjugated IgG secondary antibody and incubate for 20 min at RT.
8. Wash twice with 1 ml of HF
9. Re-suspend in 0.5-1 mL of HF for final analysis on flow cytometer.

Embedding samples in paraffin

Material :

- Neutral Buffered Formalin 10%
- 80%EtOH
- 95%EtOH
- 100%EtOH
- Xylene
- Paraffin

Procedure :

Put the cassettes with the samples in the following solutions :

1) 10% neutral buffered formalin for 1 hour
2) 10% neutral buffered formalin for 1 hour
3) 70% EtOH for1 hour (or overnight)
4) 95% EtOH for 1 hour
5) 95% EtOH for 1 hour
6) 100%EtOH for 1 hour
7) 100%EtOH for 1 hour
8) 50/50 100%EtOH/Xylene for 1 hour
9) Xylene for 1 hour
10) Xylene for 1 hour
11) Paraffin for 2 hours in the oven at 60 degrees Celsius
12) Paraffin overnight in the oven at 60 degrees Celsius

Now, we can embed the sample with the embedding machine:

1. Turn on the machine and make sure that there is enough paraffin in the two paraffin container.
2. Poor the metal mold with paraffin
3. Put the sample into the paraffin
4. Cover the metal mold with the cassette
5. Add paraffin to cover the cassette
6. Let the sample solidify on the cold plate

Haematoxylin and Eosin Staining

PURPOSE: To stain the slides with haematoxylin and eosin

Materials:

- Slides with tissue sections
- Haematoxylin solution Gill Modified solution3 (Order at VWR Harleco Cat N 65067)
- Eosin Y, solution 1% alcoholic solution (Order at VWR Harleco Cat N 588X)
- 100% EtOH
- 95% EtOH
- 80% EtOH
- Xylene
- Slides racks
- Dry dishes (there must not be water in the different dishes you use for the xylene and 100%EtOH)
- Permanent mounting media
- Coverslips
- Forceps
- Gauze sponge
- Q-tips

Procedure: the procedure involves moving the slides from solution to solution in different staining dishes

Step 1: Deparaffinizing the slides

a) Xylene for 5 minutes
b) Xylene for 1 minute
c) 100% EtOH for 1 minute
d) 100% EtOH for 30 seconds
e) 95% EtOH for 1 minute
f) 95% EtOH for 30 seconds
g) 80% EtOH for 1 minute
h) Tap water rinse for 1 minute

Step 2: Staining with haematoxylin

a) Haematoxylin 1 dip!
b) Go quickly to tap water rinse for 2 minutes
c) 1% acid solution (2mL of Acid Acetic + 98 mL of Disyilled water) : 10dips
d) Running tap water for 2 to 5 minutes

Step 3: Counterstain

1) Eosin for 2-3 minutes

Step 4: Dehydrating

a) 95% EtOH for 1 minute
b) 100% EtOH for 1 minute (use a new EtOH solution)
c) 100% EtOH for 30 seconds
d) Xylene for 1 minute (use a new xylene solution)
e) Xylene for 30 seconds
f) Leave slides in Xylene until coverslipping

Now the slides are ready to be coverslipped to preserve them.

Step 5 : To coverslip the samples to preserve them

a) Remove the slides from the xylene
b) With a gauze sponge wipe the excess clearing agent from the slide to approximately 2-3 mm from the margin of the specimen. If specimen are close to the edge of the slide, Q-tips are very useful to map up xylene
c) Using the transfer pipette, place approximately two-four drops of mounting media in the center of the slide. There must be enough medium to fill the space between the cover glass and the slide.
d) Place the edge of the cover glass on the edge of the slide over the center of the specimen
e) Gradually bring the cover glass and slide together, drawing the medium evenly over the specimen.
f) Remove wet mounting media excess with Q-tips.
g) Remove excess of mounting media with xylene.

If air bubbles occur under the cover glass tease them out by gentle tapping the cover glass with a dissection needle or forceps. If the bubbles cannot be removed soak the slide for a minute or two in clearing agent (xylene) and repeat steps 1-7.

Immunohistochemistry / Hematoxylin staining

The following procedure involves transferring the slides from one solution to the next, in sequential order.

Step 1: Deparaffinizing the slides

a) Xylene (7 min)
b) Xylene (5 min)
c) 100% EtOH (2 min)
d) 100% EtOH (2 min)
e) 1% H_2O_2 in MeOH for (20 min)
f) 95% EtOH (3 min)
g) 95% EtOH (3 min)
h) 80% EtOH for (1 min)
i) 2 x (Tap water rinse for 5 min.)
j) PBS rinse (2 min.)
k) PBS rinse (10 min.)

Prepare 1mg/ml Trypsin, 37° C

Step 2: Trypsinizing and antibody staining

a) Transfer slides to a tray with a lid; place water in tray to prevent drying of slides.
b) Dry surrounding areas of slide with gauze, careful not to touch sample.
c) Add 100μL of (1mg/mL., 37°C) trypsin to each slide. Incubate try for 15 min at room temperature (RT) with lid covering tray.
d) Rinse well with distilled water (5 min).
e) Wash in PBS (1 min).
f) Incubate at RT in normal blocking serum (20 min).
g) Blot excess serum onto KimWipe.
h) Add 1° antibody, incubate (30 min).
i) Wash slides in buffer (5 min); dry slide NOT sample.
j) Incubate in 2° antibody solution (30 min).
k) Wash slides in buffer (5 min); dry slide NOT sample.
l) Add Vectastatin ABC Reagent (30 min). (Prepare DAB solution)
m) Wash slides in buffer (5 min); dry slide NOT sample.
n) Incubate in DAB substrate solution:
o) it should take 2-10 min.
p) until the sample has a sufficiently dark (brown) color.
q) Rinse in ddH20 3X (5 min. each).

Prepare 1° antibody

Prepare *Vectastatin ABC Reagent* (incubate 30 min)

Step 3: Staining with haematoxylin

a) Rapidly dip the slides in Hematotoxylin (0.5 seconds).
b) Transfer to ddH_2O bath, and was (2 min).
c) Leave under running tap water (15 min).
d) Transfer to ddH_2O bath until next step.

Step 4: Dehydrating

a) 95% EtOH (1 min)
b) 100% EtOH (1 minute; use fresh EtOH)
c) 100% EtOH for 30 seconds
d) Xylene (1 minute; use fresh xylene)
e) Xylene for 30 seconds
f) Leave slides in Xylene until mounting.

Whole Mount EB Immunostaining

Materials:

4% formaldehyde
PBS
2% FBS diluted in PBS
0.1% Triton-X
Primary antibody
Secondary antibody
DAPI

Procedure:

1) Wash EBs at room temp twice with PBS
2) Fix EBs in 4% formaldehyde at room temperature overnight
3) Wash EBs in new 2% FBS diluted in PBS every hour for 5 hours
4) Permeabilize with 0.1% Triton-X for 1 hour
5) Wash EBs with PBS for 1 hour
6) Stain with primary antibody overnight
7) Wash 5 times with PBS over an hour
8) Stain with secondary antibody overnight
9) Wash 5 times with PBS over an hour
10) Stain with DAPI overnight
11) Wash with PBS and visualize

Alkaline Phosphatase and Von Kossa Staining

Description

Hitsochemical staining of an adherent cell population to determine the covered area of cells that exhibit the alkaline phosphatase (ALP) enzyme and those areas that have mineralized. The ALP positive areas appear red, while the mineralized areas are brown to black in colour. Bone nodules stain highly for ALP and once mature, exhibit mineralization.

Reagents

- PBS (TCMP Fac. Med. U of T)
- 10% NFB:
  - formalin/formaldehyde 100mL (Fisher Scientific, F79 1)
  - Na2HPO4 16g (Fisher Scientific, S374 1)
  - NaH2PO4.H2O 4g (Fisher Scientific, S369 1)
  - Distilled water 1L
- 2.5% Silver Nitrate solution:
  - AgNO3 2.5g (Sigma, S8157)
  - Distilled water 100mL
- Naphthol AS MX-PO4 (Sigma, N5000)
- N,N-Dimethylformamide (DMF) (Fischer Scientific, D1191)
- Tris-HCl MW=157.6 pH 8.3 0.2M
- Red Violet LB salt (Sigma F1625)
- Distilled water
- Sodium Carbonate formaldehyde:
  - formalin/formaldehyde 25mL (Fisher Scientific, F79 1)
  - Na2CO3 5g (EM Science, B10240)

- Distilled water 100mL

Method

1. Suction off medium from dish, rinse once in cold PBS.
2. Fix in 10% cold Neutral Formalin Buffer (NFB) for 15 min.
3. Suction off all buffer and rinse the dish once with distilled water, then leave in distilled water for 15 min.
4. While waiting, prepare fresh substrate:
   a. Naphthol AS MX-PO_4 0.005 g
   b. DMF (N,N-Dimethylformamide) 200 µL
   c. Tris-HCl MW = 157.6 0.2 M pH 8.3 25 mL
   d. Distilled water 25 mL
   e. Red Violet LB salt 0.03 g

In eppendorf tube, dissolve (a) in DMF. Add to graduated cylinder with (c + d). Add salt (e) to solution (a+b+c+d) and filter with Whatman's No.1 filter paper immediately prior to adding to dishes to be stained.

5. Incubate dishes for 45 minutes at room temperature.
6. Rinse in distilled water 3-4 times and leave in distilled water 1 hour (not absolutely necessary, can go to next step right after last rinse).
7. Stain with 2.5% silver nitrate for 30 min.
8. Suction off all the silver nitrate and rinse with distilled water 3 times.
9. Cover up the stained cells with distilled water for a while. Then rinse once more with distilled water to eliminate all silver nitrate remain and either examine or set to dry.

Alternative ending:

10. Prior to examination or drying dish, the colour of the mineralized nodules can be deepened by adding sodium carbonate formaldehyde to the dish for 30s – 2min. Care must be taken in the reaction time allowed as the colour may become too intense and turn dish very black, making it hard to read.
11. Suction off all the sodium carbonate formaldehyde and rinse with slowly running tap water.
12. Leave the stained cells in tap water for 1 hour and then rinse again and cover in tap water for counting or set to dry.