Appendix C

DETAILED EXPERIMENTAL PROTOCOLS

TABLE OF CONTENTS

C.1 Purification of aECM Proteins with Internal Lysines

1. Following 10 L fermentation, centrifuge in 500 mL bottles at 10,000 rpm for 15 min in tared bottles. Pellet weight should be between 200–300 g. Split pellet into two 1 L containers and redisperse in 2 ml/g TEN buffer. Freeze at –20 °C.
2. Add a small amount of DNase, RNase, and PMSF. Thaw at room temperature for 1–2 h. Transfer to 2 L flask and add water up to a final volume of 1.2 L. Stir at 4 °C overnight.
3. Bring pH up to 9 using 6N NaOH and stir for one hour. Spin in JA-14 rotor (250 mL bottles) at 14,000 rpm for 1–2 h at 4 °C. Save and record weight of pellet (P1C). Add NaCl to supernatant (S1C) to make final concentration of 1M. Stir at 4 °C overnight.
4. Incubate S1C at 37 °C for >3 h. Pre-warm JA-14 rotor to 37 °C. Spin at 14,000 rpm for 1–2 h at 37 °C. Save and record volume of supernatant (S1H). Record weight of pellet (P1H). Resuspend pellet in 1g/10mL water. Stir overnight at 4 °C.
5. Bring pH of P1H up to 9 using 1N NaOH and stir for 1 h. Spin in JA-17 rotor at 17,000 rpm for 2 h at 4 °C. Save and record weight of pellet (P2C). Add NaCl to supernatant (S2C) to make final concentration of 1M. Stir at 4 °C overnight.
6. Incubate S2C at 37 °C for >3 h. Pre-warm JA-17 rotor to 37 °C. Spin at 17,000 rpm for 2 h at 37 °C. Save and record volume of supernatant (S2H). Record weight of pellet (P2H). Resuspend pellet in 1g/10mL water. Stir overnight at 4 °C.
7. Bring pH of P2H up to 9 using 1N NaOH and stir for 1 h. Spin in JA-17 rotor at 17,000 rpm for 2–3 h at 4 °C. Save and record weight of pellet (P3C). Add NaCl to supernatant (S3C) to make final concentration of 1M. Stir at 4 °C overnight.
8. Incubate S3C at 37 °C for >3 h. Pre-warm JA-17 rotor to 37 °C. Spin at 17,000 rpm for 2 h at 37 °C. Save and record volume of supernatant (S3H). Record weight of pellet (P3H). Resuspend pellet in 1g/10mL water. Stir overnight at 4 °C.
9. Transfer P3H to dialysis tubing (25 mm, 12,000–14,000 MW cut-off). Tubing can be cleared by microwaving in EDTA solution for 3 min, rinsing with water, and repeating. Dialyze at 4 °C with 2 water changes per day for a total of 6 water changes.
10. Transfer P3H from dialysis tubing to 50 mL centrifuge tube. Centrifuge in JA-17 rotor at 17,000 rpm for 1 h at 4 °C. Save and record weight of dialyzed pellet (P4C). Supernatant (S4C) can be frozen at –20 °C and lyophilized or further temperature cycled to reduce water volume.

C.2 Bicinchoninic Acid (BCA) Quantification of Protein Adsorption

For use with: Sigma QuantiPro BCA Assay Kit (QPBCA, for 0.5–30 μg/mL protein)

1. Prepare substrates in triplicate:
    a. Adsorb 50 μL of protein solutions overnight at 4 °C in clear 96-well plates.
    b. Rinse 3× with 100 μL PBS.
2. Make protein standards. Because the assay is sensitive to the amino acid composition of proteins, make a standard for each protein to be tested. Do not use the supplied BSA standard.
    a. Make a 50 μg/mL standard: 50 μL 1 mg/mL solution + 950 μL PBS
    b. Make the following dilutions:

Concentration (μg/mL)	Vol. of 50 μg/mL standard solution (μL)	Vol. of PBS (μL)
0.5	3	297
5	30	270
10	60	240
20	120	180
30	180	120

3. For wells containing adsorbed protein, add 50 μL of PBS. Add 50 μL of each protein standard to a well. For the blank well, add 50 μL of PBS to a well containing no protein.
4. Prepare the working reagent: 4 mL QA + 4 mL QB + 0.08 mL QC
5. Add 50 μL working reagent to each well.
6. Seal plates with polyolefin tape (Nalge Nunc).
7. Incubate plates at 60 °C for 1 h.
8. Allow plates to equilibrate to room temperate for ~20 min with the sealing tape still in place.
9. Take an absorbance reading at 562 nm.

C.3 Preparation of Crosslinked, PEGylated Protein Films

1. Base-clean glass coverslips in a saturated solution of KOH in ethanol. Sonicate coverslips for 15 min. Rinse with water and ethanol and dry with canned air.
2. Make the following solution for spin-coating 5 coverslips: 72.5 μL protein solution (5 mg/mL), 26.25 μL water, and 1.25 μL BS^3 solution (10 mg/mL).
    a. Separately weigh out BS^3 for each set of coverslips.
    b. Keep protein solutions, water, BS^3, and coverslips on ice.
    c. Add water to BS^3 just before adding it to protein solution.
3. Put coverslip on spin-coater. Turn on vacuum.
4. Put 20 μL on 12 mm round coverslip (25 μL on 15 mm coverslip).
5. Spin at 4000 rpm for 45 s.
6. Store dry coverslips in a humidified chamber at 4 °C overnight.
7. Rinse three times with water.
8. Sterilize with 95% ethanol for 1 h at room temperature.
9. Rinse three times with water.
10. Make a 50 mM mPEG-SPA (MW 5000) solution.
11. Put 50 μL (60 μL for 15 mm coverslip) of PEG solution on Parafilm.
12. Dry coverslip with canned air and put it protein-side down in PEG solution.
13. React coverslip with PEG solution for 2 h at room temperature.
14. Rinse coverslips three times with water.

C.4 Antibody Staining of Crosslinked Protein Films

C.4.1 Fluorescent Imaging

1. Put coverslips in 24-well plate.
2. Rinse 3× with 1 mL of water.
3. Block with 0.5 mL of 10% BSA (not heat-inactivated) for 30 min at room temperature.
4. Add 0.25 μL of the monoclonal T7 antibody (Novagen) to the BSA solution and leave at room temperature overnight.
5. Rinse 3× with 1 mL of PBS for 5 min with no agitation.
6. Add 50 μL of the secondary antibody (Cy2-labeled anti-mouse, 0.5 mg/mL, Chemicon) + 450 μL of water per well. Incubate in dark for 1 h.
7. Rinse with 1 mL of water. Remove liquid immediately.
8. Rinse with 1 mL of water. Leave on waver for 10 min.
9. Rinse 2× with 1 mL of water for 5 min with no agitation.
10. Blot coverslips and mount on microscope slides. Seal with nail polish and store in refrigerator.

C.4.2 Western Blotting

1. Make PBS–Tween solution: 1 mL 10× PBS, 9 mL water, 10 μL Tween20
2. Make PBS–Tween–milk solution: 8 mL PBS–Tween and 0.4 g milk powder
3. Put coverslips in 24-well plate.
4. Add 500 μL of PBS–Tween–milk solution and put on waver for 1 h.
5. Add 500 μL of PBS–Tween–milk solution and 0.125 μL of anti T7 tag-HRP conjugated antibody (Novagen). Put on waver for 30 min.
6. Turn on developer.
7. Rinse coverslips with 500 μL PBS–Tween–milk and put on waver for 10 min. Repeat.
8. Rinse coverslips with 500 μL PBS–Tween and put on waver for 10 min.
9. Add equal parts of developing solution and add to coverslips for ~1 min.
10. Place coverslips in Saran wrap. Expose coverslips to film in dark room.

C.5 Cell Culture Maintenance

C.5.1 Non-enzymatic Passaging (HUVEC)

1. Warm up PBS and EGM-2 to 37 °C in the water bath.
2. Visually check cells before passaging to make sure they look close to confluent.
3. Put warm PBS and cold Versene in hood; unscrew caps.
4. Aspirate medium off of plates (only do 2 plates at a time).
5. Using 10 mL pipet, add 5 mL of warm PBS to each plate. Swirl plate and aspirate off PBS.
6. Rinse plates again with 5 mL each of warm PBS.
7. Add 5 mL of cold Versene to each plate.
8. Wait for cells to detach. If you have more plates, go ahead and do steps 4–7 to 2 plates at a time.
9. Visually check that cells look rounded and bright.
10. Rap the petri dish against the side of the table to detach cells.
11. Check that most of the cells have detached.
12. Aspirate the cells from each dish into a 15 or 50 mL tube. Use the liquid to rinse each plate before putting it into the tube.
13. Make sure you have a water balance.
14. Centrifuge the cells at 200*g* for 5 min.
15. Aspirate the liquid off the top of the cell pellet. Be sure not to disturb the pellet.
16. Resuspend the pellet using 2 mL × # of plates = mL of medium.

C.5.2 Enzymatic Passaging (CHO-α_5, COS-7, HEK 293T, HeLa, MCF-10A, MEF-GFP, MEF18)

1. Warm up PBS and DMEM to 37 °C in the water bath.
2. Rinse cells two times with 5 mL of PBS.
3. Put 1 mL of 0.5% TRED (trypsin in 0.52 mM EDTA) into each plate. Rock plate to coat entire surface. Incubate plate at 37 °C for 2–3 min.
    a. COS-7: Use 0.25% TRED for 5 min.
    b. HEK 293T: Cells lift off easily so dribble liquid down side of plate.
    c. MCF-10A: Incubate for 15 min in 0.5% TRED. To disperse cells, spin down in 20% normal horse serum.
4. Rinse plate with 1 mL of TRED to remove adherent cells. Transfer 1 mL of cells into:
    a. 2 mL serum-free DMEM with 150 µL soy bean trypsin inhibitor (30 mg/mL in PBS, Sigma T9003).
    b. 3 mL serum-containing DMEM.
5. Pellet 3 min at 1050 rpm. Aspirate off the liquid.
6. If desired, wash cells by resuspending in 3 mL of DMEM per plate and centrifuging again. This step is not necessary if cells are just being replated.
7. Resuspend in medium.

C.5.3 Counting the Cells and Making New Plates

1. Count the cells.
    a. Put 11 µL of the cell solution in the top of the hemacytometer.

b. Mix the cells with trypan blue on the side of the hemacytometer and put this solution in the bottom of the hemacytometer.
c. Using the microscope, count the number of cells in 4 squares of the top portion. Find the average.
d. In 4 separate squares in the bottom, count the number of live cells and the number of dead cells. Find the viability by adding up all the live cells in the bottom and dividing that number by the total number (live + dead) of cells counted.
e. To find the total number of cells, multiply the average from c by the viability in d by 10^4 cells/mL by the total volume in which the cells are resuspended.
f. To calculate the volume of cells that need to be added to each plate, find the concentration of the cell solution by dividing the total number of cells by the total volume of the cell solution. Now, divide # cells/plate by the cell concentration to find the volume of cells to add to each plate.

2. Fill the desired number of plates with 10 mL each of medium.
3. Add the cell solution to each plate.
4. Swirl the plates and put them in the incubator.

Cell Type	Growth Medium	# of cells to seed, days to grow	Cryovial
CHO-α_5	DMEM, 10% FBS, pen/strep, 1% non-essential amino acid solution, 1 mM sodium pyruvte	1.1×10^6 cells/plate (1:6), 2 days 2.2×10^6 cells/plate (1:12), 3 days	1 plate → 5 vials (5.3×10^6 cells/vial), 10% DMSO
COS-7	DMEM, 10% FBS, pen/strep	2×10^5 cells/plate (1:6), 3 days	1 plate → 5 vials (1×10^6 cells/vial), 5% DMSO
HEK 293T	DMEM, 10% FBS, pen/strep	1.3×10^6 cells/plate (1:15), 3 days	1×10^6 cells/vial, 10% DMSO
HeLa	DMEM, 10% FBS, pen/strep	1.5×10^5 cells/plate (1:6), 3 days	1×10^6 cells/vial, 10% DMSO
HUVEC	EGM-2	1×10^5 cells/plate, 4–5 days	3.5×10^6 cells/vial, 10% DMSO
MEF18, MEF-GFP	DMEM, 10% FBS, pen/strep	1.1×10^6 cells/plate (1:5), 2 days 5.6×10^5 cells/plate (1:10), 3 days	1 plate → 4 vials (1.4×10^6 cells/vial), 10% DMSO

Medium Components:
DMEM: Dulbecco's modified Eagle's medium (GIBCO #11965-092)
L-glutamine (GIBCO #11965-092)
10% (v/v) FBS: fetal bovine serum (GIBCO #10437-028)
pen/strep: 50 U/mL penicillin, 50 μg/mL streptomycin (GIBCO #15070-063)
1% (v/v) non-essential amino acid solution (Sigma #M7145)
1 mM sodium pyruvate (Sigma #S8636)
EGM-2: Endothelial Growth Medium-2 (Cambrex BioSciences #CC-3162)

C.6 Cell Adhesion—Centrifugation Assay for 24-well plates

1. Prepare plates
    a. Adsorb 500 μL fibronectin to wells overnight at 4 °C.
    b. Rinse wells 3× with 500 μL of water.
    c. Block wells with 500 μL of 0.2% heat-inactivated BSA for 30 min at room temperature.
    d. Rinse wells 3× with 500 μL of water.
    e. Allow cells to dry in the air.
    f. Adhere coverslips to bottom of 24-well black Visiplate (Perkin-Elmer).
    g. Rinse wells 3× with 500 μL of water.
    h. Remove water and take fluorescence reading with 485 nm excitation and 535 nm emission.
2. Passage cells
    a. Resuspend cells in 2 mL of EBM-2 (Cambrex BioSciences) per plate.
    b. Label cells with 1 μL of calcein (5 μL of DMSO + 50 μg calcein) per plate for 30 min at room temperature.
    c. Spin cells at 1050 rpm for 5 min.
    d. Rinse with 2 mL of PBS^{+} per plate.
    e. Spin cells at 1050 rpm for 5 min.
    f. Resuspend cells in PBS^{+} at a concentration of 2.67×10^{5} cells/mL.
3. Adhesion
    a. Add 1 mL of cells to each well.
    b. Incubate for 30 min.
    c. Take fluorescence reading of plates with cells.
4. Detachment
    a. Add 1 mL of Percoll (21% w/w in PBS) to each well.
    b. Centrifuge for 10 min at desired centrifugal force.
    c. Remove all liquid via pipette.
    d. Take fluorescence reading of remaining cells.
5. Calculate cell adhesion index
    a. Subtract background fluorescence from cell fluorescence.
    b. Divide cell fluorescence after centrifugation by cell fluorescence before centrifugation to obtain fraction of retained cells.
    c. Divide fraction of retained cells in test well by fraction of retained cells in control well (fibronectin, $1g$) to obtain cell adhesion index (CAI).

6. Calculate normal detachment force:
    a. Make the following assumptions
        i. Density of cell (ρ_c) = 1.07 g/mL
        ii. Density of medium (ρ_m) = 1.123 g/mL
        iii. Volume of the cell (V_m) = 0.5 pL
    b. Use Archimedes' Theorem: $F = (\rho_c - \rho_m)V_c$RCF, where F is the force, and RCF is the relative centrifugal force.
    c. Sample forces:

RCF (g)	Force (pN/cell)
1	0.26
10	2.6
100	26
500	130
1000	260
200	520
300	780

C.7 Immunofluorescence Microscopy of Integrins

1. Seed HUVECs in an 8-well chamber slide with a total volume of 500 μL. For 1–4 h samples, seed ~3×10^4 cells (75 μL of 4×10^5 cells/mL). For 24 h samples, seed ~5040 cells (12.6 μL of 4×10^5 cells/mL).
2. Rinse cells two times with 500 μL warm, filtered PBS.
3. Fix cells with 200 μL cold, filtered acetone for 1 min.
4. Rinse cells two times with 400 μL warm, filtered PBS.
5. Block cells with 200 μL filtered, non-heat-inactivated 10% BSA for 30 minutes.
6. Rinse cells two times with 400 μL filtered PBS.
7. Primary Antibody Staining: Use 100 μL of the primary antibody solution and incubate on the cells for one 1 h.

Antibody	1^o Concentration (μg/mL)	1^o Antibody (μL)	PBS (μL)
$\alpha_v\beta_3$	12.5	1.25	98.75
$\alpha_5\beta_1$	25	2.5	97.5
α_5	6.25	6.25	93.75
β_1	25	25	75
vinculin	12.5	1.25	98.75

8. Rinse three times with 500 μL of PBS, with 5 min between rinses and no agitation.
9. Secondary Antibody Staining: Use 100 μL of the secondary antibody solution and incubate cells for 1 h in the dark.

Antibody	2^o Concentration (μg/mL)	2^o Dilution	2^o Antibody (μL)	Phalloidin (μL)	10 % BSA (μL)	PBS (μL)
$\alpha_v\beta_3$	12.5	1:80	2.5	3.8	30	63.7
$\alpha_5\beta_1$	6.25	1:160	1.25	3.8	30	64.95
α_5	6.25	1:160	1.25	3.8	30	64.95
β_1	3.125	1:320	0.625	3.8	30	65.575
vinculin	12.5	1:80	2.5	3.8	30	63.7

10. Rinse the cells once with 500 μL of PBS. Immediately rinse again with 500 μL of PBS and wait for 10 min while agitating the slide on the waver. Rinse two more times with 500 μL of PBS with 5 min in between rinses and no agitation.
11. DAPI Staining: Use 200 μL of a 3×10^{-7} M DAPI solution for 15 min.
    a. Make two 100-fold dilutions of the 10.9 mM solution (10 μL solution: 990 μL PBS) to make a 10.9×10^{-7} M solution.
    b. Make the 3×10^{-7} M DAPI solution: 55 μL of 10.9×10^{-7} M solution: 145 μL PBS.
    c. Rinse three times with 500 μL of PBS.
12. Remove chamber walls, blot slide, add filtered mounting medium, put coverglass on top, and seal with nail polish.

C.8 Cell Migration

1. Block each well of a 6-well plate with 1 mL of 0.2% heat-inactivated BSA (fraction V, Sigma) for 30 min at room temperature.
2. Rinse wells three times with 2 mL of water.
3. Allow wells to air dry.
4. Apply autoclaved Dow Corning high vacuum grease (VWR #59344-055) around the edge of the coverslip bottom.
5. Adhere coverslip to bottom of the well. Make sure that the grease is along the whole edge of the coverslip, otherwise water will seep into the space between the coverslip and the well.
6. Rinse wells three times with 2 mL of water.
7. Using a heated needle, make a hole in the left side of well 1.
8. Insert the 1/16" end of the barbed fitting (barbed fitting, clear PP 1/8" to 1/16", Cole-Palmer #U-06365-44) into the hole.
9. Tape the barbed fitting into place and use vacuum grease to seal the hole on the underside of the cover.
10. Fill well 1 with water to prevent evaporation of media in the other wells. Fill other wells with 3 mL of EGM-2.
11. Add cells (1.6×10^4 to 6×10^4 cells per well) and put the plate in the incubator. Allow cells to adhere for 1–2 h.
12. While cells are adhering, set up environmental chamber around the microscope.
    a. The environmental chamber is constructed out of cardboard that is covered with Reflectix, a foil insulation that looks like mylar-covered bubble wrap. The edges of the Reflectix should be covered with tape so that the foil does not get into the electronics. The chamber should enclose the stage and the lenses to prevent thermal drift but should not include heat sources such as the lamp.
    b. Connect the ducting from the heat source to the environmental chamber. The heater has an environmental temperature control (Ranco) and the probe should be taped down to the microscope inside of the chamber.
    c. Seal the environmental chamber using duct tape.
    d. Turn heater on and allow to equilibrate to 37 °C for ~1 h.
13. Put a thin layer of mineral oil (embryo tested, Sigma #M8410) on top of the EGM-2 to prevent evaporation.
14. Use Teflon tape to seal the edge where the well-plate and the cover meet.
15. Put 6-well plate on microscope stage.
16. Connect gas tubing to 1/8" end of barbed fitting. Gas should come from gas tank, go through a gas washing bottle (to humidify the gas and prevent evaporation), and then connect to barbed fitting on 6-well plate.
    a. Gas tank: 5% CO_2, 20% O_2, balance N_2, medical grade from Air Liquide with the CGA 500 fitting
    b. Regulator: Concoa Regulator, 0–15 psi delivery pressure, 1/4 MNPT, CGA 500 (Air Liquide # 315-1311-5)
    c. Needle valve: Air Liquide #555-3225 (The valve is important for maintaining a steady flow rate overnight.)

d. Connector: purchased from stock room to change valve size from 1/4" to tubing size
e. Gas washing bottle: purchased from glass blowing shop but available as standard laboratory glassware

17. Sequentially open valves on gas tank, regulator, and needle valve. Adjust gas flow rate so that bubbles are vigorously being formed in gas washing bottle. The flow rate should not be too high otherwise evaporation may occur.
18. Use Metamorph Basic Imaging Software v. 6.2r1 (Molecular Devices, Downingtown, PA) to save various locations and use timelapse-scan feature to record images every 15 min for 24 h.
    a. On Menu bar, press Acquire from Flashbus button. To see image, press Start Live. Use joystick to find desired location.
    b. Go to Devices → Stages → Move Stage to Absolute Position.
    c. To save a position, press Memorize… and press Memorize again.
    d. To see a list of memorized positions, press Memory List…
        i. To move to a particular location, highlight the position and press Move.
        ii. To delete a position, highlight it and press Edit… Highlight the position again and press Cut Position to delete.
        iii. To edit the x, y, or z coordinates of a location, highlight it and press Edit… Highlight the position again, press Edit Position… and press Use Motor Position to change coordinates. Press OK.
        iv. When memory list is complete, save it by pressing Save… and giving it a file name.
    e. On Menu bar, press Timelapse Scan. Select the correct magnification and press OK. Choose the time lapse interval and length (default is 15 min interval for 24 h) and press OK.

C.9 Cell Migration Analysis

C.9.1 Converting Tiff Files to Quicktime Movies

1. Open ImageJ 1.30v (National Institutes of Health, Bethesda, MD) in OS X.
2. Go to File → Import → Image Sequence…
3. Highlight first tiff file of a location and press Open.
4. In the Sequence Options dialog, the settings should be:
    a. Number of images: should be automatically detected
    b. Starting image: enter the position number
    c. Increment: enter the number of total positions
    d. File name contains: leave blank
    e. Convert to 8-bit grayscale: leave unchecked
    f. Open ½ size: leave unchecked
5. Go to File → Save As → Quicktime Movie…
6. Choose a name and location for the Quicktime movie and press Save.
7. In the Compression Settings dialog, the settings should be:
    a. Compression type: video
    b. Frames per second: 8
    c. Key frame every 10 frames: checked
    d. Quality: medium

C.9.2 Analyzing Quicktime Movies

1. Go to System Preferences → Startup Disk (under System). Select Mac OS 9.2.2 on OS 9 and press Restart… Make sure the DIAS dongle is plugged into the computer before restarting.
2. Open Dynamic Image Analysis System (DIAS) 3.2 (Solltech, Oakdale, IA).
3. Go to File → Open… and select desired Quicktime movie.
4. In the Verify Time-base dialog, the settings are:
    a. Unit of time: sec
    b. Frame rate: 8
5. Go to → DIAS → Auto Trace by Threshold… and set the desired range of frames to analyze.
6. If desired, select region by mouse or analyze whole image.
7. In the Auto Trace by Threshold dialog, use the following settings. Use the preview buttons to see how the settings affect the outlines and the arrow keys to change frames.
    a. Threshold: cells range from 100–215 (pick value based on your images)
    b. Min pixels: 40
    c. Max pixels: 750
    d. Dilate: 1
    e. Erode: 1
    f. Smooth image: 0
    g. Smooth outline: 1
    h. Resolution: normal
    i. Invert image: unchecked
    j. Remove halos: unchecked

8. Go to DIAS → Trace on Movie. Check that cells have been correctly outlined. If you only track cells that are present in the first frame, you do not need to worry about extraneous outlines in subsequent frames. However, if you decide to track cells whose first appearance is in a later frame, it is often easier to erase the outlines that are not of interest in all of the frames. Make sure that all outlines are completely closed. Some useful commands are:
    a. 1–6: magnifies the image
    b. mouse button: draws outline
    c. alt + mouse button: erases outline
    d. c: clears all tracings
    e. u: undo
    f. v: finish and close current curve
    g. n: next frame
    h. b: previous frame
    i. g: goto frame
    j. q: done
9. Go to DIAS → Backup Tracing… to save tracing. The file ending should be .bktrace.
10. Go to DIAS → Make Path from Trace… Use the following settings:
    a. First frame: 1
    b. Last frame: last frame of cells traced
    c. Frame increment: 1
    d. Max movement/frame: 100
    e. Min # pixels/object: 5
    f. Min path length: 0
    g. Allow new objects: usually leave unchecked
    h. Allow static objects: usually check
    i. Allow edge contact: unchecked
    j. Indicate initial objects: unchecked
11. Do not include interior pixel data in the path file. Press enter to accept paths.
12. Save path file in appropriate location. The file name will end in .path. Any changes to the path will result in a file name that ends in .edit or .edit2.
13. In the Edit Path File Header dialog, these are the settings to use:
    a. Title: enter name of substrate and location
    b. Date: automatically entered
    c. Trial: 1
    d. Time unit: min
    e. Frame rate: 0.0667 fr/min (corresponds to one frame every 15 min)
    f. Distance unit: um
    g. Scale factor: 1.26689 um/pixel
    h. Screen size: 640 × 480
    i. Pre-spline resolution: 33%
    j. Post-spline resolution: 100%
    k. Spline bias: 1
    l. Spline tension: 1
    m. Expert spline adjustment: unchecked

n. Use area-based centroids: check

14. Check that paths are unbroken and cells appear and disappear in appropriate frames. Useful commands include:
    a. ViewPaths → Select Object… and enter object number to view one or multiple cells at a time.
    b. Cmd + n: next frame
    c. Cmd + b: previous frame
    d. Cmd + g: goto frame
    e. EditPaths → Delete Path… and enter object number to delete.
    f. EditPaths → Join Fixed Path… and enter paths to join. This command is useful if paths were broken.
15. Go to DIAS → Compute Parameters… and select the appropriate path file.
16. To get cell migration speed, change the following settings:
    a. Summary: check
    b. All data: check
    c. Plot: uncheck
    d. First frame: frame to start (starting on the second frame gives an average cell speed that does not include 0 for the first frame)
    e. Last frame: frame to end
    f. Speed: check
    g. Use central differences method: uncheck
17. Save summary as a tab-delimited format for Excel. This file will be saved in the same location as the path file with the name (cst->data).text. You can change the file name to whatever you choose.
18. Open Microscoft Excel and go to File → Open… At the bottom of the dialog box, change Files of type setting to All Files (*.*) and select appropriate text file. Press finish on the Text Import Wizard. At the top of the file, the average cell speed will be listed for each object.

C.10 Fluorescence Imaging of Newly Synthesized Proteins in Mammalian Cells

C.10.1 Preparation of Substrate

1. Adsorb 200 μL fibronectin solution (10 μg/mL) in each well of a Lab-Tek II Chamber Slide (8-well, Nalge Nunc International, Rochester, NY) at 4 °C overnight.
2. Rinse wells three times with 200 μL PBS.
3. Block wells with 200 μL of a 2 mg/mL heat-inactivated BSA solution (fraction V, Sigma) for 30 min at room temperature.
4. Rinse wells three times with 200 μL PBS.

C.10.2 Preparation of Cells

1. Rinse cells two times with 5 mL of PBS.
2. Put 1 mL of 0.5% TRED (trypsin/EDTA) into each plate. Rock plate to coat entire surface.
3. Incubate plate at 37 °C for 2–3 min.
4. Rinse plate with 1 mL of TRED to remove adherent cells. Transfer 1 mL of cells into 2 mL DMEM with 150 μL soy bean trypsin inhibitor (30 mg/mL in PBS, Sigma T9003).
   - DMEM (pH 7.7): DMEM (Sigma, D0422) without methionine, glutamine
     50 mL total 0.584 g/L glutamine= 1 mL of 200 mM = 4 mM final conc.
     1 mg/mL BSA= 50 mg per 50 mL
5. Pellet 3 min at 1050 rpm. Aspirate off the liquid.
6. Wash cells by resuspending in 3 mL of DMEM per plate and centrifuging again.
7. Resuspend in medium.
8. Count cells and distribute in 8-well slides.
   a. MEF-GFP, MCF-10A: 2.5×10^4 cells/well
   a. COS-7, HeLa: 5×10^4 cells/well
   b. CHO-α_5, HEK 293T, HUVEC: 10^5 cells/well

C.10.3 Incorporation of Met or Hpg

1. Add protein synthesis inhibitor if desired. Incubate 30 minutes at 37 °C.
   - 50 μM of cycloheximide (dissolved in water): 3 μL of 5 mM stock to each well containing 300 μL total
   - 40 μM of anisomycin: 1.5 μL of 8 mM stock to each well containing 300 μL total
2. Add filtered amino acid solution:
   - 1 mM HPG: 6 μL of 50 mM stock solution to 300 μL
   - 1 mM Met: 6 μL of 50 mM stock solution to 300 μL
3. Incubate for 4 h.
4. Chase with 1 mM Met for 2 h.
   - Remove medium.
   - Wash once with 300 μL PBS.
   - Add 300 μL DMEM supplemented with 1 mM Met.

C.10.4 Washing and Fixing Cells

1. Rinse cells three times with 300 μL warm, filtered PBS, pH 7.4.
2. Fix cells with 300 μL filtered 3.7% paraformaldehyde solution for 10 minutes.
   - 3.7% paraformaldehyde
     i. Put 0.3 g paraformaldehyde in glass vial.
     ii. Add 1.5 mL distilled water.
     iii. Add 3 drops of 1–2 N NaOH.
     iv. Heat to 60 °C for 15–30 min with intermittent shaking until solid has dissolved.
     v. Add 6.6 mL of PBS.
     vi. Bring pH to ~7.5 with HCl.
     vii. Filter with 0.2 μM filter.
3. Rinse cells two times with 300 μL warm, filtered PBS, pH 7.4.
4. For nucleoli staining, permeabilize with 300 μL of 0.1% Triton X100 for 3 min. Rinse two times with 300 μL of PBS.
5. Block with 100 μL of blocking solution (10% fetal calf serum, 5% sucrose, 2% BSA) for ≥30 min at room temperature. Rinse 2–3 times with PBS.
6. Stain for nucleoli after rinsing off blocking solution.
   - Chemicon Antibody
     i. Primary (1:40) = 35 μL of 1° antibody + 1365 μL of PBS
     ii. Secondary 12.5 μg/mL (1:80) : 40 μL of 2° antibody + 480 μL of 10% BSA + 1080 μL of PBS
7. Rinse cells three times with 300 μL filtered PBS, pH 7.4.

C.10.5 Coumarin Labeling of Fixed Cells

1. Add "click reaction mix" to wells. Total volume of ≥1.2 mL/well is needed for the Lab-Tek II chamber slide.
   - Click Reaction Mix:
     22.5 μL fresh ligand (stock= 200 mM)
     18 μL TCEP (stock= 0.5M, freshly made)
     45 μL $CuSO_4$ (stock= 100 mM)
     56.25 μL 3-azido-7-hydroxycoumarin (stock= 10mM)
     22.5 mL PBS (pH 7.5)
     * Final Concentrations: 200 μM ligand, 400 μM TCEP
     200 μM $CuSO_4$, 25 μM coumarin
2. The wells were sealed with tape and inverted.
3. Wells were reacted overnight at room temperature on the waver.

C.10.6 Washing and Visualizing Finished Slides

1. Rinse cells four times with 300 μL of filtered PBS, 1% Tween buffer, 0.5 mM EDTA. The last three washes had one minute incubation while being agitated.
2. Rinse once with water.

3. Remove chamber walls, blot slide, add filtered mounting medium, put coverglass on top, and seal with nail polish. Take fluorescent/DIC images using 510 inverted with two photon laser.
   The DIC settings for the 63× are:
   a) Prism: 100× slider "III PlanApo 1.4"
   b) Aperture: 1.2117
   c) NA: 0.5 (near condenser on left side of microscope)
   d) Filter at 0°
   e) Filter on DIC II (note: III also works)
   f) Gain: 365
   g) Offset: –1.615
   h) Slider: screwed all the way out

C.11 Labeling of Newly Synthesized Proteins in Mammalian Cells for Flow Cytometry

C.11.1 Seeding Cells (3 days before labeling experiment)

1. Seed cells into individual dishes:
    a. 35 mm: 1.2×10^5 cells, 1.5 mL total volume, 9.6 cm^2
    b. 60 mm: 2×10^5 cells, 4 mL total volume, 28.0 cm^2
    c. 100 mm: 5.6×10^5 cells, 10 mL total volume, 78.5 cm^2
2. Be sure to seed control plates:
    a. MEF (no GFP): react in PBS only
    b. MEF (no GFP): react in click mix
    c. MEF-GFP: react in PBS only

C.11.2 Labeling Cells

1. Remove liquid from 35 mm plates. Wash cells twice with 2 mL (4 mL for 60 mm plates) of warm PBS. Swirl plates in between. Add <1 mL (<2 mL for 60 mm) of DMEM (pH 7.7) to each plate.
2. Add protein synthesis inhibitor if desired:
    - 50 μM of cycloheximide (dissolved in water): 10 μL of 5 mM stock to each 35 mm plate containing 1 mL total
    - 40 μM of anisomycin: 5 μL of 8 mM stock to each 35 mm containing 1 mL total
3. Wait for 30 min, add filtered amino acid solution, and incubate for 4 h:
    - 1 mM HPG: 10 μL of 100 mM stock solution to 0.99 mL
    - 1 mM Met: 7.46 μL of 134 mM stock solution to 0.9925 mL
4. Chase for 2 h: Remove DMEM. Rinse once with PBS. Add new DMEM supplemented with 1 mM Met (made 50 mL stock = 373.1 μL 134 mM Met to 50 mL).
5. After pulse/chase: Wash cells twice with 2 mL (4 mL for 60 mm) of warm PBS. Detach using 250 μL TRED (500 μL for 60 mm). Incubate at 37 °C for 2–3 minutes. Add detached cells to three times the volume of serum-containing medium.
6. Centrifuge at 200*g*, 3 min.
7. Resuspend in 730 μL of PBS and then add 270 μL of 3.7% paraformaldehyde (to make 1% paraformaldehyde). Fix cells for 10 min.
8. Centrifuge and wash with 1 mL of PBS. Centrifuge again.
9. Block with 250 μL of blocking solution for ≥30 min at room temperature.
10. Centrifuge solution and add click reaction mix (500 μL) for reaction overnight at 4 °C. *For control (MEF) reactions, add PBS to samples.*
11. Wash once with 1 mL of PBS, 1% Tween buffer, 0.5 mM EDTA. Resuspend in 500 μL of PBS.
12. Filter using 50 μm Nytex nylon membrane.