Appendix 2: Detailed Protocols

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Photolithographic Patterning of Proteins on Glass Coverslips

This protocols produces coverslips patterned over the majority of their surface area. There are still some batch to batch irregularities, but generally the quality is pretty good.

1. Acid Cleaning
    a. Set an 800 mL beaker in a bath of ice with a stirbar on a stirplate, in a fume hood. Wear a lab coat, goggles, and gloves and use the splash shield if available.
    b. Pour 75 mL of cold 30% hydrogen peroxide into the 800 mL beaker.
    c. While stirring, **SLOWLY** add 175 mL concentrated sulfuric acid to above solution.
    d. Mark coverslips to distinguish top from bottom, an etched L works fine
    e. Load coverslips in wafer basket
    f. Carefully remove stirbar from acid solution and add wafer basket.
    g. Allow to soak for 1 hr
2. Remove Wafer Basket from Acid Bath and Rinse
    a. 3 x 5 minutes water
    b. 1 x 5 minutes 95% ethanol
3. Silanize
    a. Mix 2% solution of APTES in 95% ethanol (8 mL APTES, 20 mL water, plus 372 mL 95% ethanol).
    b. Allow APTES solution to hydrolyze for 5 minutes.
    c. Put coverslips in Aptes for 10 minutes.
4. Remove Coverslips from Silane Solution and Rinse.
    a. 4 x 5 minutes 95% ethanol
5. Cure APTES layer by heating coverslips at ~100° C for 15 minutes.
6. Remove from oven and allow to cool.
7. Derivatize APTES Layer.
    a. Add 10 mg 4-benzoylbenzoic acid, succinimidyl ester to 1 mL DMSO
    b. Put blank coverslips on wafer trays.
    c. Place 90 uL drops of DMSO solution on each coverslip.
    d. Remove APTES coverslips from wafer basket and place on top of DMSO drop, L mark up, to form a coverslip sandwich.
    e. Leave overnight (~12 hours).
8. Transfer Coverslips to Wafer Basket.
9. Rinse 4 x 5 minutes 95% ethanol.
10. Rinse coverslips in PBS and store in the dark at 4° C until photopatterning.
11. Photopattern (in Michael Roukes' cleanroom on the mask aligner)
    a. Put 50 uL drop of protein solution (~1 mg/mL) on photo mask
    b. Place derivatized coverslip on drop, L mark up
    c. Expose to UV for 2-10 minutes, assuming a luminosity of 10 mW/cm^2.
    d. Transfer coverslips to PBS vial.

e. Repeat as necessary.
f. Rinse mask.

12. Rinse and Mount Slides
    a. Rinse 3 x 5 minutes PBS.
    b. Load coverslip in culture chamber.
    c. Apply 0.5-2 mL of fibronectin at 10ug/mL for 1 hour. Volume depends on size of coverslip and chamber.
    d. Rinse 2x PBS.
13. Coverslip is ready for cell culture.

Materials:	**Manufacturer**
1. Conc. Sulfuric Acid	EM Sciences (VWR) SX1244-6
2. 30% Hydrogen Peroxide	EM Sciences (VWR) HX0635-2
3. 95% Ethanol	EM Sciences (VWR) EX0280-3
4. Aminopropyltriethoxysilane	Sigma A-3648
5. 4-benzoylbenzoic acid, succinimidyl ester	Molecular Probes B-1577
6. Dimethylsulfoxide	EM Sciences (VWR) MX1457-6
7. Wafer Basket:	Fluoroware A14-018-0216
8. Wafer Basket Cover	Fluoware A14-028-0215
9. Wafer Basket Handle	Fluoware A029-0215 12"
10. Wafer Tray/Individual Coverslip Storage	Fluoware H22-15-0615
11. Wafer Tray Cover	Fluoware H22-151-0615

Alternate Photolithography Protocol

Example of a representative successful protocol for patterning coverslips, prior to optimization for simple organic solvents. All reagents are the same as previous patterning protocol. All organic solvents are reagent grade from EM Sciences. Coverslips generated with those protocol were not compatible with neural crest explant culture.

1) Soaked coverslips in Scavenger solution (210 mL HCl (36-38%) 90 mL H202 (30%)) for 2 hrs
   a) Rinse in flowing H20.
   b) Rinse 2 x 15 minutes ddH20 (800 mL)
   c) Rinse 1 x 15 minutes EtOH (400 mL)

2) Silanized coverslips overnight in 2% APTES/EtOH (total volume: 0.4L)

3) Washed coverslips

a)	1 x 10 minutes	Toulene	500 mL
b)	1 x 10 minutes	EtOH	500 mL
c)	1 x 10 minutes	pH9.5 H20	800 mL
d)	1 x 10 minutes	Toluene	500 mL
e)	1 x 10 minutes	EtOH	500 mL
f)	1 x 10 minutes	CH2Cl3	300 mL
g)	1 x 10 minutes	Pentane	300 mL

4) Crosslinked coverslips overnight w/150 uL (10mg B1577 in 1 mL DMSO) at room temperature.

5) Rinsed all coverslips

a)	1 x 10 minutes	CH2Cl3	300 mL
b)	1 x 10 minutes	Toluene	500 mL
c)	1 x 10 minutes	Pentane	500 mL

6) Exposed to UV for 30 minutes w/ 50 uL of 1 ug/uL protein solution.

7) Rinsed all coverslips

a)	1 x 15 minutes	pH 9.0 Na Bicarb 0.1 M	300 mL
b)	1 x 15 minutes	1 M NaCl	500 mL
c)	1 x 15 minutes	ddH20	500 mL

Chlorosilane Patterning Protocol from Chris Chen, Johns Hopkins University

1) Acid wash coverslips as per general photolithography protocol (A2-2).
2) Rinse in ddH2O.
3) Bake at 90°C for 20 minutes.
4) Allow to cool.
5) Place in clean vacuum dessicator.
6) Add 1 drop of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies T-2492) to the bottom of the desiccator. The chlorosilane will irreversibly silanize any glass, so use disposable glass pipettes to transfer the solution. 1 drop will coat all of the glass inside a desiccator, so there is no need to carefully measure it.
7) Apply a static vacuum and wait 1 hour to overnight.
8) Apply a silicone mold to the glass.
9) Apply a light vacuum to the mold.
10) Suck a drop of 50:50 ethanol:water through the mold.
11) Suck a drop of 25:75 ethanol:water through the mold.
12) Suck 3 drops of water through the mold.
13) Suck a drop of protein (1-5 ug/mL) into the mold.
14) Wait 2-3 hours.
15) Suck 3-5 drops of water through the mold.
16) Submerge the mold in water, then remove it.

Quail Neural Crest Cultures – (Based on Protocol By Rusty Lansford)

1. Put quail eggs in shaking incubator big end up, 44-52 hours prior to culture.

Preparation: (Best done in laminar flow hood or other clean area)

2. Turn on blower/UV hours in advance.
3. Wipe down working area with 70% ethanol.
4. Wash hands carefully.
5. Wipe down everything entering hood with 70% ethanol.
6. Mix either Neural Crest Media or Defined Media.
7. Mix up dispase solution.
8. Add 1% Penn/Strep to sterile Howard Ringer's Solution.
9. Add fibronectin to substrate (glass or plastic) in 35 mm tissue culture dish, at 10-20 ug/mL.
10. Let substrate stand for 45 minutes to an hour at room temperature, then pipet off excess fibronectin solution, rinse once with PBS, and add culture media.

Tools to lay out in flow hood with dissecting scope:

- Scissors
- Serrated forceps
- Sharp tungsten needle probe
- Fine forceps
- 3 mL syringe with 18 gauge needle
- 1 mL syringe with 25 gauge needle (bent to moderate angle)
- 1/10 India ink/Ringer's
- Petri dish with Ringer's

Start of Explant Procedure

11. Remove eggs from incubator and spray with 70% ethanol.
12. Allow eggs to stand at room temp for 30 minutes.
13. Prepare waste bag for eggs.
14. Fill ice bucket; put sterile 3 well glass dish on ice.
15. With 3 mL syringe remove 0.5-1 mL of albumin from each egg.
16. Open egg with scissors.
17. Inject small amount of ink under embryo (IF NEEDED).
18. Using serrated forceps and scissors, cut embryo out of egg.
19. Transfer embryo to dish filled with Ringer's solution.
20. Repeat for all 40 eggs.
21. Clean up embryos, using forceps to hold embryo and edge of tungsteen needle to cut out the trunk region.
22. Transfer trunk segments to cold 3 well glass dish with long sterile glass pipet, being careful not to allow trunks to enter body of pipet.

23. Remove most of the Ringer's solution.
24. Add dispase solution, mix trunk segments, remove dispase, and add fresh dispase solution.
25. Immediately put dish on ice; leave on ice for 15 minutes.
26. Add serum blocking solution to wells 2 and 3.
27. Pipet trunks gently up and down in dispase solution.
28. Watch trunk segments for signs of dissociation and change dispase regularly. Total time of pipetting trunk segments should be 5-15 minutes.
29. As neural tubes separate transfer them quickly to well #2, transferring minimal volume between wells.
30. Once all neural tubes are in well #2, transfer selectively to #3, checking to make sure that no notocords are still attached. Rinse in dispase again if needed.
31. Rinse neural tubes through 3 changes of serum blocking media, then 3 changes of appropriate serum free culture media.
32. Add neural tubes to culture dishes and return dishes to incubator.
33. Mechanically remove neural tubes 6-18 hours after start of incubation.

Standard Solutions

Dispase: 3 mg/mL Dispase II in 20 mM Hepes buffered DMEM, (made fresh)

Dissecting Solution: Howard Ringer's solution with 1% Penn/Strep

a) Add 7.2 g NaCl, 0.23 g CaCl, 0.37 g KCl to 1/2 L ddH2O.
b) Add 10.5 mL of dibasic sodium phosphate and 2.6 mL monobasic potassium acid phosphate to solution. (Stock dibasic is 0.946 g in 100 mL ddH2O and stock monobasic is 0.907 g in 100 mL ddH2O).
c) Add 400 mL ddH2O.
d) Adjust pH to 7.4 then fill to 1 L.

Serum Blocking Solution (neutralizes Dispase):

i. 44 mL of DMEM
ii. 5 mL Serum (usually fetal bovine serum; type not important)
iii. 1 mL 1M Hepes
iv. 0.5 mL Penn/Strep

Neural Crest Media: F12 Basal Media (contains L-glut), 1% Penn/ Strep, 1% N2 Supplement

Defined Media: DMEM High Glucose (contains L-glut), 1% Penn/Strep, 1% NEAA

Imaging Media: Neural Crest Media + 20 mM Hepes buffer.

Materials:	Manufacturer
1. Quail Eggs	AA Laboratories
2. India Ink (Fount India)	Koh-I-Noor 9150-1
3. DMEM, high glucose, plus L-glutamine	Irvine Scientific 9031
4. Hepes buffer solution, 1M	Irvine Scientific 9319
5. Penicillin-Streptomycin (Penn/Strep)	Irvine Scientific 9366
6. Fetal Bovine Serum	Irvine Scientific 3000
7. F-12 Nutrient Mixture (Ham)	Gibco/BRL 11765-054
8. Dispase II (neutral protease, grade II)	Boehringer Manheim 165 859
9. Human Fibronectin	BD Labware 4008B

Optical Tweezers Experimental Outline

Day 1

1) Set 40 quail eggs in plastic trays at 37°C, 44-52 hours prior to intended neural tube prep.

Day 2

1) Bead Derivatization: **(See protocol sheet A2-11)**
2) Silanize beads in aminopropyltriethoxysilane solution in ethanol.
    a. Rinse 3x in PBS
    b. Biotinylate in EZ-Link-Imino-Biotin
    c. Rinse 3x in PBS
    d. Streptavidinate beads.
    e. Rinse 3 times in PBS.
3) Protein Derivatization
    a. Incubate in protein mixture.
        i. negative control
        ii. biotin-Protein-G
    b. Rinse 3x in PBS
    c. Incubate half of negative control and Protein-G in ephrin-FC protein mixture.
    d. Rinse 3x in PBS

Day 3

1) Coverslip preparation (**See protocol sheet A2-13**)
    a. Acid wash
    b. Silanize
    c. Cure
    d. Coat in protein
2) Culture chamber assembly
    a. 40 mm coverslips in 6 well dishes
3) Neural tube prep **(See protocol sheet A2-6)**
    a. Surgically extract embryos from eggs.
    b. Surgically isolate trunk region of interest.
    c. Enzymatically isolate neural tube from trunk region.
    d. Treat with serum.
    e. Culture on coverslip chambers.

Day 4

1) Evaluate outgrowth
2) Mechanically remove neural tubes.
3) Assemble cell culture chamber with microenvironment on tweezers stage.
4) Experiment
    a. Film normal behavior (5-15 minutes)
    b. Stimulate cells with coated beads.
    c. Film reaction.

Materials:	**Manufacturer**
1. Glass beads, 1.58 um diameter	Duke Scientific 8150
2. Aminopropyltriethoxysilane	Sigma A-3648
3. EZ-Link NHS-Iminobiotin (Pierce)	Fisher Scientific 1117AJ
4. Streptavidin	Molecular Probes S-888
5. Protein G-Biotin Conjugate	Calbiochem 203198
6. Mouse ephrin-B1/FC chimera	R&D Systems 473-EB-200
7. 40 mm coverslips, 250/pk	BiopTechs 40-1313-0319
12. Conc. Sulfuric Acid	EM Sciences (VWR) SX1244-6
13. 30% Hydrogen Peroxide	EM Sciences (VWR) HX0635-2
14. 95% Ethanol	EM Sciences (VWR) EX0280-3
15. Aminopropyltriethoxysilane	Sigma A-3648
16. Dimethylsulfoxide	EM Sciences (VWR) MX1457-6
17. Wafer Basket:	Fluoroware A14-018-0216
18. Wafer Basket Cover	Fluoware A14-028-0215
19. Wafer Basket Handle	Fluoware A029-0215 12"
20. Wafer Tray/Individual Coverslip Storage	Fluoware H22-15-0615
21. Wafer Tray Cover	Fluoware H22-151-0615
22. Imaging Media: See A2-7.	

Neutravidin Bead Coating Protocol (Based on Protocol by Kevin Thigpen)

1. Make up 2 batches of 2% aminopropyltriethoxysilane in dry ethanol (20 uL APTES in 1 mL EtOH), each in a 1.7 mL eppendorf.
2. Mix bead solution well, then transfer 100 uL of beads to each APTES/ethanol solution.
3. Silanize for 30 minutes at room temperature.
4. Centrifuge for 1 minute at 6 krpm.
5. Pipette away solution and discard.
6. Rinse 3x with PBS.
    a. Add 1 mL PBS to tube.
    b. Mix extensively.
    c. Centrifuge for 1 minute at 6 krpm.
    d. Pipette away solution.
7. Weigh out 6 mg of Pierce EZ Link NHS-LC-Biotin. Add 200 uL dry DMSO, then 800uL PBS.
8. Add 500 uL DMSO solution to each tube of beads.
9. Biotinylate for 10 minutes at 37° C.
10. Centrifuge for 1 minute at 6 krpm.
11. Pipette away solution and discard.
12. Rinse 3x with PBS.
    a. Add 1 mL PBS to tube.
    b. Mix extensively.
    c. Centrifuge for 1 minute at 6 krpm.
    d. Pipette away solution.
13. Add 200 ug streptavidin in 0.5 mL PBS to the tube.
14. Streptavidinate for 30 minutes at 37° C.
15. Centrifuge for 1 minute at 6 krpm.
16. Pipette away solution and discard.
17. Rinse 3x with PBS.
    a. Add 1 mL PBS to tube.
    b. Mix extensively.
    c. Centrifuge for 1 minute at 6 krpm.
    d. Pipette away solution.
18. Add 50 ug of Protein G in 0.5 mL PBS to tube.
19. Incubate for 30 minutes at 37° C.
20. Centrifuge for 1 minute at 6 krpm.
21. Pipette away solution and discard.
22. Rinse 3x with PBS.
    a. Add 1 mL PBS to tube.
    b. Mix extensively.
    c. Centrifuge for 1 minute at 6 krpm.
    d. Pipette away solution.
23. Add 200 uL of Imaging Media to Protein-G beads and store.

24. Add 20 ug of ephrin-B1 to 1 eppendorf of Protein-G beads.
25. Incubate for 30 minutes at 37°C.
26. Centrifuge for 1 minute at 6 krpm.
27. Pipette away solution and discard.
28. Rinse 3x with PBS.
    a. Add 1 mL PBS to tube.
    b. Mix extensively.
    c. Centrifuge for 1 minute at 6 krpm.
    d. Pipette away solution.
29. Add 200 uL of Imaging Media to ephrin-B1 beads and store.

Fibronectin Substrates for Optical Tweezers Experiments

1) Load 40 mm #1 glass coverslips (Bioptechs) into a Fluoware wafer basket.
2) Add 500 mL of 95% ethanol to a 1 L beaker, add wafer basket with coverslips to beaker and sonicate for 5 minutes.
3) Silanize coverslips in 2% aminopropyltriethoxysilane (APTES) solution (10 mL APTES, 25 mL water, 465 mL ethanol) for 15 minutes.
4) Rinse 3 x 500 mL 95% ethanol for 5 minutes each.
5) Cure at 65-80°C for 20 minutes to an hour.
6) Store under static vacuum until needed.
7) Before neural tube prep (A2-6), add a 400 uL drop of 20ug/mL human fibronectin to the bottom of a 60 mm petri dish. Place coverslip on top. Incubate at room temp for 1 hour.
8) Transfer coverslip to fresh 60 mm tissue culture dish, inverting the coverslip so that the fibronectin side faces up.
9) Add 5 mL of neural crest media (A2-7) and incubate at 37°C until needed.

Best-fit Sphere Analysis of *Xenopus Laevis* Embryos

All spherical coordinate analysis of the *Xenopus* volumetric data was implemented and executed in Matlab (The Mathworks Inc., Natick MA). Typically only one of the three 8-bit color channels was processed to reduce memory load. In order to estimate the best-fit sphere, the image volume was initially down-sampled by a factor of six in all dimensions and smoothed by a 0.65 sample radius Gaussian filter. The interior and exterior of the embryo were segmented by conversion to a binary image using a threshold determined by Otsu's Algorithm (1) prior to 2D hole filling using internal functions provided by Matlab. The isosurface mesh representing the external boundary of the embryo was generated from the segmented binary data again using internal Matlab functions. A least-squares best-fit sphere was calculated for the mesh vertices using a custom implementation of the algorithm suggested by David Eberly (2). The coordinates of the center and the radius parameterize the best-fit sphere, so the cartographic coordinates of the sphere surface require specification of a pole and prime meridian, both of which can be supplied by an experienced user. For this application, the "south pole" is defined as the center of the blastopore at the embryo surface and the prime meridian is the midline axis of symmetry of the archenteron. Once the blastopore center has been located, the down-sampled data is re-sampled along planes of constant latitude and redisplayed to allow the center of the archenteron to be specified. All further re-sampling can now be performed in the blastopore-archenteron spherical frame of reference. Shell

segments from within the high-resolution data were extracted by supplying ranges of radius, latitude and longitude to a custom rapid tri-linear interpolator implemented in C and interfaced to Matlab. The stacks of extracted shell segments are essentially three-dimensional (radius, latitude, longitude) image data which can be exported to other visualization applications or redisplayed using the Matlab Mapping Toolbox in various cartographic projections.

References

1. Otsu N. Threshold Selection Method from Gray-Level Histograms. Ieee Transactions on Systems Man and Cybernetics 1979; 9:62-66.
2. Eberly D. Least-squares Fitting of Data. http://www.magic-software.com/Documentation/LeastSquaresFitting.pdf.

Acknowledgements

Code Design: J. Michael Tyszka and Andrew J. Ewald

Code Implementation: J. Michael Tyszka

Classic Stripe Assay (Protocol from Rusty Lansford)

Store silicone matrices are stored in 70% ethanol

Preparation: All done in tissue culture hood unless noted.

1. Take matrices out of 70% ethanol and allow to dry.
2. UV sterilize 35 mm tissue culture dishes for 2 hours.
3. Cut out 2.5 cm^2 of nitrocellulose filter. Dissolve in 10 mL methanol.
4. Add 100 uL of nitrocellulose solution to each dish, and rotate dish so that nitrocellulose coats uniformly.
5. Insert a small piece of PE-10 tubing into matrix fill channel.
6. Make up solutions (all solutions made up in PBS):
    a. Antibody Solution: 250 ug/mL goat anti-FC antibody, 100 ug/mL human fibronectin, 10 ug/mL BSA-Texas Red.
    b. Fibronectin Solution: 250 ug/mL human fibronectin.
    c. Ligand-FC Solution: 10 ug/mL ligand-FC
7. Filter sterilize all solutions through 0.2 micron filter.
8. Attach silicon matrices to culture dishes, making to sure to get a good seal.
9. Outline the extent of the matrix on the bottom of the dish with a Sharpie.
10. Take 1 mL of Antibody Solution in 1 mL plastic syringe, add fine gauge needle to syringe, and connect to tubing in matrix. Push 1/3 of solution through the matrix.
11. Wait 20 minutes.
12. Push 1/3 of solution through the matrix.
13. Wait 20 minutes.

14. Push 1/3 of solution through the matrix
15. Wait 20 minutes.
16. Push 1/2 mL PBS through matrix.
17. Remove matrix from dish and wash 3x with PBS.
18. Coat entire dish with Fibronectin Solution for 1 hour (typically 1.5 mL).
19. Remove PBS.
20. Add 1.5 mL Ligand-FC solution to dish.
21. Wait 2 hours.
22. Rinse 3x with PBS.
23. Add neural crest media.
24. Culture quail neural tubes as usual (See A2-6).

Materials:	**Manufacturer**
1. Goat Anti-Human IgG	Jackson Immunoresearch, 109-005-098
2. Human Fibronectin	BD Labware, 4008B
3. BSA-Rhodamine	Sigma, A-2289
4. Silicon Matrices	Juergen Jung, (MPI Entwicklungsbiologie)
5. PBS: Cell Culture Grade	Sigma, D8662
6. Nitrocellulose Paper	Schleicher & Schuell 20570

Movie	Cell	Logic	Protein	Beads	Cell Contact	Collapses	Polarity Changes	Score 0-2
A	1	No Stim	-	-	0	0	0	
	2	No Stim	-	-	0	2	1	
B	1	No Stim	-	-	3	1 (bleb)	0	
	2	No Stim	-	-	2	0	0	
	3	No Stim	-	-	3	0	0	
C	1	No Stim	-	-	0	0	0	
	2	No Stim	-	-	0	0	0	
D	1	No Stim	-	-	0	0	1	
E	1	No Stim	-	-	3	1	3	
	2	No Stim	-	-	4	0	2	
	3	No Stim	-	-	2	1	2	
	4	No Stim	-	-	4	0	0	
	5	No Stim	-	-	3	1	3	
F	1	No Stim	-	-	2	1	1	
	2	No Stim	-	-	2	0	0	
2	1	Leading Edge	ephrin-B1	1	1	0	1	1
3	1	Leading Edge	ephrin-B1	3	0	1	1	2
4	1	Seq Leading Edge	ephrin-B1	2 cluster	0	2	2	2
5	1	Tripod Leading Edge	ephrin-B1	1	0	1	1	1
6	1	Leading Edge	ephrin-B1	1	0	0	3	1
7	1	Leading Edge	Protein-G	1	1	1	0	0
	2	No Stim	Protein-G	-	1	1	0	0
8	1	Leading Edge	Protein-G	1	0	1	1	1
11	1	Multi-Stim	ephrin-B1	7	0	2	3	2
12	1	Leading Edge	ephrin-B1	4	0	1	1	2
	2	Multi-Stim	ephrin-B1	2	0	1	1	2

Movie	Cell	Logic	Protein	Beads	Cell Contact	Collapses	Polarity Changes	Score 0-2
13	1	No Stim	ephrin-B1	-	1	0	2	0
	2	Leading Edge	ephrin-B1	1	1	1	2	2
14	1	Leading Edge	ephrin-B1	2	1	2	3	2
	2	No Stim	ephrin-B1	-	1	2	2	0
15	1	Leading Edge	ephrin-B1	1*	0	1	1	1
16	1	Multi-Stim	Protein-G	3	0	3	4	2
17	1	Multi-Stim	Protein-G	4	0	0	1	0
18	1	Leading Edge	Protein-G	1*	0	0	0	0
19	1	Seq Leading Edge	Protein-G	1+cluster	0	0	1	0
20	1	Lateral Edge	ephrin-B1	1	0	1	1	1
21	1	Lateral Edge	ephrin-B1	1	1	0	0	0
	2	Lateral Edge	ephrin-B1	4	1	0	0	0
22	1	Lateral Edge	ephrin-B1	Cluster	0	1	1	2
23	1	Seq Leading Edge	ephrin-B1	2	1	2	2	2
	2	Lateral Edge	ephrin-B1	2	1	2	1	1
	3	Multi-Stim	ephrin-B1	4	1	1	4	1
24	1	Lateral, then Lead	ephrin-B1	4	0	2	2	1
25	1	Lateral Edge	ephrin-B1	2	0	1	1	1
26	1	Lateral Edge	Protein-G	3	0	1	2	1
27	1	Lateral Edge	Protein-G	1	0	1	1	1
28	1	Lateral Edge	Protein-G	6	0	1	2	2
29	1	Lateral Edge	Protein-G	2	0	0	0	0
30	1	Multi-Stim	ephrin-B1	11	0	0	0	0
31	1	Leading Edge	ephrin-B1	4	0	3	4	2
32	1	Leading Edge	ephrin-B1	cluster	0	2	3	2
33	1	Leading Edge	ephrin-B1	1 + cluster	0	1	1	2

Movie	Cell	Logic	Protein	Beads	Cell Contact	Collapses	Polarity Changes	Score 0-2
34	1	Leading Edge	ephrin-B1	1 cluster	1	2	1	2
35	1	Seq Stim	ephrin-B1	2 + cluster	1	0	2	2
	2	Leading Edge	ephrin-B1	1	1	0	1	0
36	1	Multi-Stim	ephrin-B1	4 + cluster	0	1	1	1
37	1	Multi-Stim	Protein-G	4	1	2	3	2
	2	No Stim	Protein-G	-	1	0	0	0
38	1	Leading Tripod	Protein-G	cluster	0	1	3	2
39	1	Leading Edge	Protein-G	5	1	2	2	2
	2	Multi-Stim	Protein-G	4	2	2	3	2
40	1	Leading Edge	Protein-G	4	0	1	1	2
41	1	Leading Edge	Protein-G	2	0	0	0	0
45	1	Leading Edge	ephrin-B1	1	0	1	1	1
46	1	Leading Edge	ephrin-B1	cluster	0	2	2	2
47	1	Leading Edge	ephrin-B1	2	0	0	1	1
48	1	Leading Edge	ephrin-B1	3	0	0	1	1
49	1	Leading Edge	ephrin-B1	1	0	1	2	1
	2	Leading Edge	ephrin-B1	1	0	2	2	2
50	1	Leading Edge	ephrin-B1	1	0	2	2	2
51	1	Leading Edge	ephrin-B1	1	0	0	0	0
52	1	Leading Edge	Protein-G	1	2	1	1	1
53	1	Leading Edge	Protein-G	1	0	1	2	1
54	1	Leading Edge	Protein-G	1 + cluster	0	1	2	2

Movie	Cell	Logic	# of Beads	Protein	Collapses		Polarity		Score 0-2	
					Koos	Ewald	Koos	Ewald	Koos	Ewald
3	1	Leading Edge	3	**ephrin-B1**	**0**	**1**	**1**	**1**	**1**	**2**
4	1	Seq Leading Edge	2 cluster	**ephrin-B1**	**2**	**2**	**2**	**2**	**2**	**2**
5	1	Tripod Leading Edge	1	**ephrin-B1**	**0**	**1**	**0**	**1**	**0**	**1**
6	1	Leading Edge	1	**ephrin-B1**	**0**	**0**	**0**	**3**	**0**	**1**
8	1	Leading Edge	1	Protein-G	0	1	1	1	0	1
11	1	Multi-Stim	7	**ephrin-B1**	**1**	**2**	**1**	**3**	**1**	**2**
12	1	Leading Edge	4	**ephrin-B1**	**1**	**1**	**1**	**1**	**1**	**2**
	2	Multi-Stim	2	**ephrin-B1**	**0**	**1**	**0**	**1**	**0**	**2**
15	1	Leading Edge	1*	**ephrin-B1**	**1**	**1**	**1**	**1**	**1**	**1**
16	1	Multi-Stim	3	Protein-G	1	3	1	4	2	2
17	1	Multi-Stim	4	Protein-G	0	0	0	1	0	0
18	1	Leading Edge	1*	Protein-G	0	0	0	0	0	0
19	1	Seq Leading Edge	1+cluster	Protein-G	0	0	0	1	0	0
20	1	Lateral Edge	1	**ephrin-B1**	**1**	**1**	**1**	**1**	**1**	**1**
22	1	Lateral Edge	Cluster	**ephrin-B1**	**1**	**1**	**1**	**1**	**2**	**2**
24	1	Lateral, then Lead	4	**ephrin-B1**	**0**	**2**	**0**	**2**	**0**	**1**
25	1	Lateral Edge	2	**ephrin-B1**	**1**	**1**	**0**	**1**	**0**	**1**
26	1	Lateral Edge	3	Protein-G	0	1	1	2	0	1
27	1	Lateral Edge	1	Protein-G	0	1	0	1	0	1
28	1	Lateral Edge	6	Protein-G	1	1	1	2	0	2
29	1	Lateral Edge	2	Protein-G	0	0	0	0	0	0
30	1	Multi-Stim	11	**ephrin-B1**	**0**	**0**	**0**	**0**	**0**	**0**
31	1	Leading Edge	4	**ephrin-B1**	**1**	**3**	**1**	**4**	**1**	**2**
32	1	Leading Edge	cluster	**ephrin-B1**	**2**	**2**	**2**	**3**	**2**	**2**
33	1	Leading Edge	1 + cluster	**ephrin-B1**	**0**	**1**	**0**	**1**	**1**	**2**
36	1	Multi-Stim	4 + cluster	**ephrin-B1**	**0**	**1**	**0**	**1**	**0**	**1**
38	1	Leading Tripod	cluster	Protein-G	1	1	1	3	1	2
40	1	Leading Edge	4	Protein-G	1	1	1	1	2	2
41	1	Leading Edge	2	Protein-G	0	0	0	0	0	0
45	1	Leading Edge	1	**ephrin-B1**	**1**	**1**	**1**	**1**	**1**	**1**
46	1	Leading Edge	cluster	**ephrin-B1**	**1**	**2**	**2**	**2**	**2**	**2**
47	1	Leading Edge	2	**ephrin-B1**	**0**	**0**	**0**	**1**	**0**	**1**
48	1	Leading Edge	3	**ephrin-B1**	**0**	**0**	**1**	**1**	**2**	**1**
49	1	Leading Edge	1	**ephrin-B1**	**1**	**1**	**1**	**2**	**1**	**1**
	2	Leading Edge	1	**ephrin-B1**	**1**	**2**	**1**	**2**	**2**	**2**
50	1	Leading Edge	1	**ephrin-B1**	**1**	**2**	**1**	**2**	**1**	**2**
51	1	Leading Edge	1	**ephrin-B1**	**0**	**0**	**0**	**0**	**0**	**0**
53	1	Leading Edge	1	Protein-G	1	1	1	2	0	1
54	1	Leading Edge	1 + cluster	Protein-G	1	1	1	2	2	2