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Corresponding Author:	Claire Robertson Lawrence Livermore National Laboratory Livermore, California UNITED STATES
Corresponding Author's Institution:	Lawrence Livermore National Laboratory
Corresponding Author E-Mail:	clairejanerobertson@gmail.com
Order of Authors:	Camilla Hochman-Mendes
	Tatiana Coelho-Sampaio
	Ariel Kent
	Jamie L Inman
	Mina J Bissell
	Claire Robertson
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TITLE:

Generating a Fractal Microstructure of Laminin-111 to Signal to Cells

AUTHORS AND AFFILIATIONS:

Camilla Hochman-Mendez[1], Tatiana Coelho-Sampaio[2], Ariel J. Kent[3], Jamie L. Inman[3], Mina J. Bissell[3], Claire Robertson[4]

1: Regenerative Medicine Research, Texas Heart Institute, Houston, Texas
2: Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil
3: Division of Biosciences, Lawrence Berkeley National Lab, Berkeley, California
4: Materials Engineering Department, Lawrence Livermore National Lab, Livermore, California

KEYWORDS:

Extracellular matrix microstructure, polyLM, Laminin-111, fractal, extracellular matrix

SUMMARY:

We describe three methods to generate Ln1 polymers with fractal properties that signal to cells differently compared to unpolymerized Ln1.

ABSTRACT:

Laminin-111 (Ln1) is an essential part of the extracellular matrix in epithelia, muscle and neural systems. We have previously demonstrated that the microstructure of Ln1 alters the way that it signals to cells, possibly because Ln1 assembly into networks exposes different adhesive domains. In this protocol, we describe three methods to generate polymerized Ln1.

INTRODUCTION:

Unlike growth factors or cytokines, extracellular matrix (ECM) proteins can assemble into structural networks. As ECM dysfunction is a key element of many disease conditions, the mechanisms by which cells sense and transduce signals from ECM composition, microstructure and biomechanics are attractive targets for therapeutic development. Growing evidence suggests that microstructure of these networks plays a major role in how these proteins signal to cells. To date, this linkage between ECM microstructure and cell function has been shown for collagen I[1], fibronectin[2], and fibrin[3].

Laminin-111 (Ln1) is a trimeric, cross-shaped protein that is a key structural component of the extracellular matrix that contacts epithelial cells[4], muscle cells[5], and neural cells[6]. In the mammary gland, Ln1 is necessary for functional differentiation of mammary gland epithelial cells into milk producing cells [7-9], and Ln1 is crucial for induction of tumor reversion[10,11]. Ln1 and other laminins are necessary for development of cortical actin networks and sarcolemma organization in muscle[12,13], and for neural cell migration and neurite outgrowth[13] . Thus, understanding the mechanisms by which laminin signals to cells is an active area of research.

Ln1 contains multiple adhesive domains for a broad range of receptors including integrins, syndecans, dystroglycan, LBP-110 and LamR (**Figure 1A**)[4,14]. The E8 fragment, which contains the

c-terminus globular domains of laminin α1, is necessary for binding dystroglycan and integrins α6β1 and α3β1[15], and it is necessary for functional differentiation of epithelial cells[15,16]. In contrast, the short arms show different biological activity[17], meaning that availability for recognition of these different Ln1 domain after network formation could alter cell behavior.

We have recently demonstrated that Ln1 arranged into a polymeric network exhibits fractal properties (i.e., a structure that is self-similar across multiple length scales)[18]. Fractal networks signal differently to epithelial cells compared to Ln1, which lacks this arrangement[19]. This fractal network indicates a particular assembly structure, where the long arm is preferentially exposed to cells[18]. As a result of this different long arm display, epithelial cells undergo functional differentiation into milk producing cells with well-organized tight junctions and suppression of actin stress fibers[19].

We describe 3 methods to generate Ln1 networks from short arm-short arm binding interactions, which show a high display of the Ln1 long arm: 1) by cell-free assembly with acidic buffers, 2) by co-incubation with glycoproteins, or 3) by interaction with cell surface dystroglycan. These three methods generate similar laminin microstructures through three very different mechanisms, permitting flexibility in experimental design. Previous work suggests that these three methods could represent biological robustness: in mammary gland epithelia, either cell surface dystroglycan, glycoproteins, or artificially structured laminin can induce functional differentiation[19]. Thus, researchers can choose between these methods based on the study subject: dystroglycan expressing cells show no sensitivity to Ln-1 microstructure, as dystroglycan in the cell membrane induces correct polymerization into a fractal network[19,20]. Matrigel, a mix of laminins and glycoproteins[21], represents the most economical source of Ln-1, and offers the necessary microstructure to induce dystroglycan knockout mammary cells to differentiate[20], but contains a complex mixture of proteins with lot to lot variability. PolyLM represents the cleanest, most reductionist system that provides this function, but at increased cost and lower efficacy to induce mammary cell differentiation compared to Matrigel[19].

These three methods have a fractal network structure characteristic of diffusion limited aggregation[18,19], and show altered biological activity compared to aggregated laminin in multiple experimental systems[22-25]. Future studies using these methods could identify the receptors and signaling necessary for epithelial differentiation and determine to restore normal cell-matrix interactions in cells in abnormal microenvironments[20], such as tumors.

PROTOCOL:

1. Thaw and aliquot laminin-111

NOTE: Purified laminin-111 is available commercially (typically purified from EHS matrix sold as Matrigel), but not all sources provide intact, nondegraded protein.

1.1. Confirm that Ln1 is not degraded by sourcing Ln1 and running size exclusion chromatography[26] or polyacrylamide gel electrophoresis[26]. Denatured Ln1 should have bands at

337 kDa, 197 kDa and 177 kDa, and native Ln1 should have a single band at 711 kDa.

1.2. Thaw Ln1 on ice in a refrigerator overnight, and aliquot into low protein binding microcentrifuge tubes (typically aliquot at 100 µg/tube for culture overlays, or 10 µg for coatings) using low protein binding tips and sterile technique[27]. Freeze aliquots and store at -80 °C.

2. **Laminin polymerization using low pH buffers**

2.1. Make polyLM polymerization buffer: Weigh out and mix 1 mM $CaCl_2$ and 20 mM sodium acetate in ultrapure water. Then adjust the pH to 4.0, using HCl or acetic acid. Sterile filter through 0.2 µm filter, and store at 4 °C.

2.2. Make control polymerization buffer: Mix 1 mM $CalCl_2$ and 20 mM Tris and adjust the pH to 7.0, using 10 N HCl or 12 N NaOH as necessary. Sterile filter through 0.2 µm filter, and store at 4 °C.

NOTE: Protocol can be paused here.

2.3. Thaw the needed number of Ln-1 aliquots in a refrigerator overnight.

2.4. Using sterile cell culture technique, add the appropriate buffer (either polyLM polymerization buffer from step 2.1 or control polymerization buffer from step 2.2) to laminin aliquots at a final concentration of 100 µg/mL (i.e. add 0.5 mL to 50 µg aliquots) and mix gently by pipetting.

2.5. If using fluorescently tagged laminin for visualization, reconstitute and add at 1:50 wt/wt ratio (e.g. 2 µg/100 µg) and mix gently by pipetting.

NOTE: If using laminins as a substrate for cell attachment, follow steps 2.6-2.8:

2.6. Immediately after dilution in appropriate buffer, transfer laminins onto culture plastic or glassware and incubate overnight in a cell culture incubator at 37 °C. Note that when coating coverslips, add a sufficiently large volume as to produce an equilibrated drop (typically, use 50 µL for a 13 mm round coverslip), and keep in a humidified chamber overnight at 37 °C.

2.7. Using sterile technique[27], wash with 1x phosphate buffered saline (PBS).

2.8. Remove the liquid carefully. Do not allow surface to be completely dry.

NOTE: If using laminins to cover cells for milk protein induction, follow steps 2.9-2.12:

2.9. Culture mammary epithelial cells. Culture MepG dystroglycan knock in (DgKI) or MepG dystroglycan knockout cells (DgKO) cells in DMEM-F12 supplemented with 2% FBS, 0.01 mg/mL insulin, 0.005 µg/mL EGF, 0.05 mg/mL gentamycin, and 0.05 g/mL normocin.

2.10. Seed DgKO and DgKI cells for laminin stimulation at 10,500 cells/cm^2 in well plates or coverslip bottom dishes

2.11. Incubate diluted laminins in a cell culture incubator at 37 °C for 30 min.

2.12. Centrifuge polyLM aliquots at 2,000 x *g* for 20 minutes, but centrifuge LM in neutral pH at 20,000 x *g* for 20 minutes due to the smaller size of the protein entities and need for higher speeds to collect effectively.

2.13. Using sterile technique[27], remove the supernatant gently, and resuspend in an appropriate volume of induction medium of DMEM-F12 supplemented with 3 µg/mL prolactin, 2.8 µM hydrocortisone, and 5 µg/mL insulin

2.14. Transfer laminin in cell culture medium immediately to stimulate cells. We have successfully stimulated cells with 50-800 µg/mL Ln1

3. Laminin polymerization using isolated glycoproteins

3.1. Thaw recombinant nidogen-1 and Ln1 gently on ice in the refrigerator overnight.

3.2. Mix Ln1 with nidogen-1 at a 1:1 wt/wt ratio in cell culture medium using low protein binding tips and tubes and using sterile technique. Use pure Ln1 in cell culture medium as a control.

3.3. If using fluorescently tagged laminin for visualization, reconstitute and add at 1:50 wt/wt ratio (e.g., 2 µg/100 µg). Gently mix by pipetting.

3.4. Incubate Ln1-nidogen or control Ln1 in a cell culture incubator at 37 °C for 30 min.

3.5. Centrifuge Ln1-nidogen copolymers at 2,000 x *g* for 30 min and the control Ln1 at 20,000 x *g* for 30 min.

3.6. Gently remove the supernatant and resuspend in cell culture medium to a final concentration of 100-800 µg total protein/mL.

3.7. Use immediately to stimulate cells, or transfer to coverslips and allow to adhere overnight at 37 °C in humidified cell culture incubators.

3.8. Remove culture medium from coverslips, wash coverslips with 1x PBS, and then fix with 10% formalin (i.e., 4% formaldehyde in PBS) for 15 minutes at room temperature.

4. **Laminin polymerization using dystroglycan expressing cells**

4.1. Culture mammary epithelial cells. Culture MepG dystroglycan knock in (DgKI) or MepG

dystroglycan knockout cells (DgKO) cells in DMEM-F12 supplemented with 2% FBS, 0.01 mg/mL insulin, 0.005 µg/mL EGF, 0.05 mg/mL gentamycin, and 0.05 g/mL normocin.

4.2. Seed DgKI cells at 5,000 cells/cm^2, and seed DgKO cells at 8,000 cells/cm^2 due to differences in growth rates. Culture at 37 °C in humidified incubators with media changes every 2-3 days and passages every 4-5 days using a rigorous sterile technique. Count cells carefully with a hemocytometer or automated cell counter[27] to ensure appropriate growth rates.

4.3. Seed cells for laminin stimulation at 10,500 cells/cm^2 in well plates or coverslip bottom dishes and culture for 2 days, using DgKI cells to generate fractal Ln1 and DgKO cells as negative controls. Thaw Ln-1 gently overnight on ice in a refrigerator, and then mix with induction medium of DMEM-F12 supplemented with 3 µg/mL prolactin, 2.8 µM hydrocortisone, and 5 µg/mL insulin. For a 35 mm dish, use 1 mL of induction media with 50-800 µg of Ln1.

4.4. If using fluorescent Ln1, mix in at 50:1 wt/wt ratio.

4.5. Remove media from cells, and cover with laminin and induction media of DMEM-F12 supplemented with 3 µg/mL prolactin, 2.8 µM hydrocortisone, and 5 µg/mL insulin

4.6. Culture cells for 2 days, and then fix with 10% formalin.

5. Characterization of laminin microstructure via optical microscopy

5.1. Collect fixed samples and wash 3 times with 1x PBS at room temperature for 5 min.

5.2. Block and permeabilize samples in 1x PBS with 0.5% Triton X-100, 3% bovine serum albumin, 10% goat serum, and 40 µg/mL anti-mouse blocking antibody fragment for 1 h at room temperature in a humidified chamber.

NOTE: Triton X-100 is hazardous. Handle with gloves and eye protection and dispose of appropriately.

5.3. Mix anti-laminin antibody at a dilution of 1:100 with 1x PBS with 0.5% Triton X-100 and 3% BSA and add to samples for 2 h at room temperature or overnight at 4 °C in a humidified chamber (typically use tip boxes with a wet laboratory wipe in the bottom).

5.4. Remove primary antibody and wash 3 times with 1x PBS with 0.5% Triton X-100 and 3% BSA.

5.5. Add an appropriate secondary antibody at a dilution of 1:500 to samples in 1x PBS with 0.5% Triton X-100 and 3% BSA and incubate at room temperature in the dark in a humidified chamber.

5.6. Remove secondary antibody and wash 3 times with 1x PBS with 0.5% Triton X-100 and 3%

BSA.

5.7. For samples containing cells, add 6 µM phalloidin for 45 min, followed by 3 washes with PBS with 0.5% Triton X-100 and 3% BSA, and followed by 3 washes with PBS. Then stain with 0.1 µg/mL DAPI in PBS for 5 minutes.

5.8. Mount samples if appropriate.

5.9. Image ln-1 structure using high resolution confocal microscopy. Use a laser scanning microscope equipped with immersion objectives (water immersion Plan Apochromat 40X/1.1NA or oil immersion Plan Apochromat 63X/1.4NA).

6. Characterization of laminin constructs via box counting dimension

6.1. Analyze for fractal properties using box counting dimension algorithms available in Matlab toolboxes or in ImageJ. These methods threshold images of laminin and plot on a log-log plot the number of boxes containing Ln1 compared to the size of the boxes. The slope of the relationship between these parameters is the box-counting dimension: non-fractal geometries will have a dimension of 0, 1, or 2, whereas fractal geometries have a non-integer dimension[28].

NOTE: Acid-treated, glycoprotein-treated and DgKI cell-attached Ln1 all have a box-counting fractal dimension of ~1.7, whereas control Ln1 has a fractal dimension of 2.0.

7. Characterization of laminin constructs via electron microscopy

7.1. Resuspend laminins in cell culture media and allow to adsorb to silica wafers for 90 min in a humid 37 °C environment.

7.2. Fix matrices in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2.

NOTE: Glutaraldehyde is hazardous and should be handled in a fume hood with gloves and eye protection and disposed of as hazardous waste.

7.3. Postfix matrices in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at pH 7.2.

NOTE: Osmium tetroxide is hazardous and should be handled in a fume hood with gloves and eye protection and disposed of appropriately. Cacodylate is hazardous and should be handled in a fume hood with gloves and eye protection and disposed of appropriately.

7.4. Wash 3 times in sodium cacodylate buffer at pH 7.2.

7.5. Dehydrate with increasing concentrations of ethanol.

7.6. Sputter coat matrices with a thin layer of gold.

7.7. Image using scanning electron microscopy.

REPRESENTATIVE RESULTS:

Laminin-111 is a trimeric protein with three self-assembling domains at the end of its short arms (**Figure 1A**). When treated suitably, the short arms can polymerize into a hexagonal lattice (**Figure 1B,1C**), which displays diffusion-limited aggregate fractal properties at larger spatial scales (**Figure 2**). Laminin incubated in calcium-containing neutral-pH buffers forms small aggregates visualized with an anti-Ln1 antibody (**Figure 2A**), which have a box counting fractal dimension of ~2, indicating no fractality. In contrast, Ln1 incubated in calcium-containing pH4 buffer (**Figure 2B**), or in neutral buffer with nidogen-1 (**Figure 2C**) form space filling lacy networks with a fractal dimension of ~1.7 (**Figure 3**). Ln1 cultured with dystroglycan expressing cells forms similar lacy networks (**Figure 2D**, inset shows cell nuclei).

When pH7Ln and polyLM are imaged with SEM with increasing resolution, the differences in microstructure become more apparent. At low resolution (**Figure 2Ei and v**), polyLM networks appear more spread compared to pH7-Ln, and at high resolution, pH7-Ln aggregates are apparent (**Figure 2E iv**) whereas a spread hexagonal lattice can be seen in polyLM at high resolution (**Figure 2E viii**).

FIGURE AND TABLE LEGENDS:

Figure 1: Background of Ln1 and polymerization method: A: Ln1 is a complex trimeric protein with multiple adhesive domains (receptor binding sites noted), which can bind a variety of integrin and non-integrin receptors (in black) and form polymeric networks by short arm-short arm binding (in tan). B: Ln1 can form a hexagonal lattice in the presence of calcium ions and either low pH, glycoproteins such as nidogen-1 or cell surface dystroglycan. C: example SEM image of polymerized Ln1 showing hexagonal lattice and proposed Ln1 assembly model.

Figure 2: Representative data for Ln1 polymerization. A: Ln1 aggregates in neutral buffers with calcium. When visualized with an anti-Ln1 antibody, small aggregates can be seen. B: Ln1 in acidic calcium containing buffer polymerizes into polyLM, which shows fractal properties. C: Ln1-nidogen-1 mixture (1:1 wt/wt) forms similar polymers. D: Ln1 in neutral buffered cell culture media overlaid on dystroglycan expressing cells shows a similar lacy network. Inset: nuclear counterstaining showing cell morphology. E: Scanning electron microscopy of pH7-Ln and polyLM networks (spin down method) with increasing resolution. In pH7-Ln, low resolution scans (i & ii) show relatively compact fibers, which resolve as aggregates in high resolution scans (iii). polyLM at the same concentration is much more spread (iv&v), and high-resolution scans show a hexagonal, space filling lattice (vii). F: This microstructural change causes DgKO mammary gland epithelial cells to functionally differentiate into milk producing cells[19]. i: pH7-Ln stimulated cells show abundant actin stress fibers (red) and disorganized zo-1 containing tight junctions (green), whereas ii: polyLM stimulated cells contain less total filamentous actin, lateral localization of actin into the cell-cell boundary and well organized zo-1 containing tight junctions.

Figure 3: Fractal dimension estimation with the box counting method. A&B: Raw image of pH7-

Ln and polyLM show differences in morphology. C&D: these images are thresholded to give rise to a black and white image, E&F: then the log of the ratio of the number of boxes containing the image to the size of the box is calculated. The average slope represents the box counting dimension Fd is calculated. pH7-Ln has a characteristic dimension of 2.0, indicating no fractal properties, whereas polyLM has a dimension of ~1.7, indicative of a diffusion limited aggregation process.

DISCUSSION:

Laminins represent a key element of the ECM in epithelial, muscular, and neural organ systems, and have been shown in vitro to play essential roles in regulating the functional differentiation of cells. Growing evidence indicates that the structure of laminin displayed to cells regulates its signaling and resultant cell phenotype[15,16], thus methods to control Ln1 microstructure should be of interest to basic biologists working in these fields, and to tissue engineers seeking to recapitulate normal behavior in vitro. More generally, the microstructure of a variety of extracellular matrix proteins is known to regulate biological response and understanding the mechanisms by which microstructure modulates cell function is an active area of research.

In this work, we describe three methods to polymerize laminin into networks with fractal microstructure characteristic of diffusion limited aggregation. These Ln1 polymers have been shown to signal differently to cells compared to aggregated Ln1, potentially due to altered adhesive domain display to cells. These three methods to control Ln1 microstructure may represent biological redundancy in assembly of Ln1, but this represents a challenge for experimental work. Controlling Ln1 microstructure using acidic buffer treatment will only show effects when added to dystroglycan knockout cells[19,20]. Thus, experimental design must consider the cells and the purity of Ln1 to be used. Commercially available Ln1 is commonly derived from EHS matrix, which contains a high percentage of nidogens and other glycoproteins; Ln1 in EHS matrix polymerizes correctly into fractal networks in neutral buffered systems. Recombinant Ln1 has recently become available commercially but is exceptionally costly.

Ln1 protein quality is crucial for this work as short arm fragments can block polymerization. Short arm fragments (i.e., the E4 fragment) interfere with network formation as each fragment induces a void in the network[29]. Previous data shows that inclusion of E4 fragments can block Ln1 induced phenotypes[29,30]. Likewise, we have found that network formation is disrupted by small quantities of laminin-332 (unpublished data), which like E4 fragments contains a single adhesive domain[29,30]. Thus, it is crucial to check that Ln1 is intact, and highly purified to remove other laminin species before starting work.

While these methods are relevant to researchers studying cell-matrix interactions, it should be noted that the laminin family is highly complex, involving 15 known members and additional splice variants[31], and this family interacts with a wide range of receptors, glycoproteins, collagens and potentially growth factors[4]. Thus, polymerized Ln1 represents a reductionist state which can be expected to be modified rapidly by cell function. Furthermore, different organ systems sense, respond to, and remodel laminins differently: we have demonstrated that linkage of laminin to actin through dystroglycan is dispensable for the mammary gland[19], whereas dystroglycan is

absolutely necessary for myocyte function[32].

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DISCLOSURES:

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Figure 1

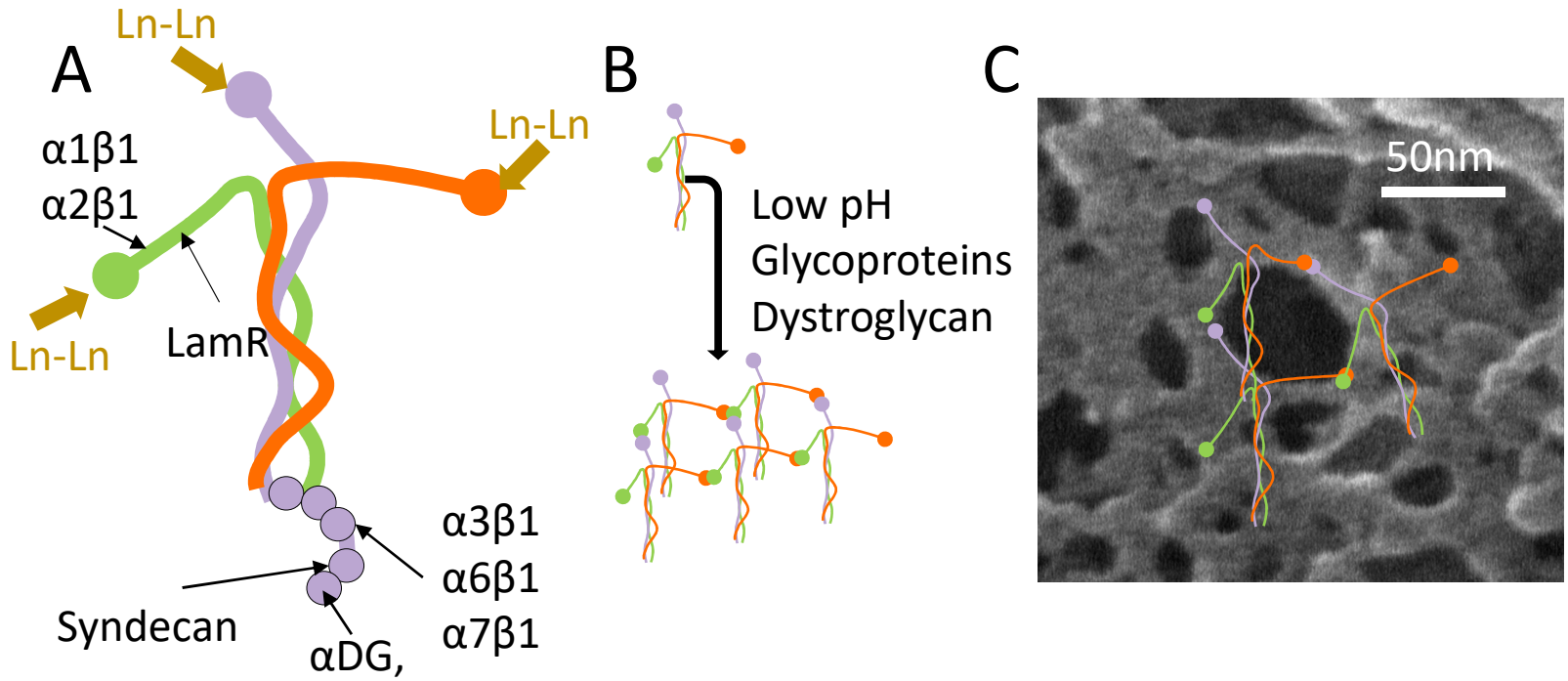

Figure 2

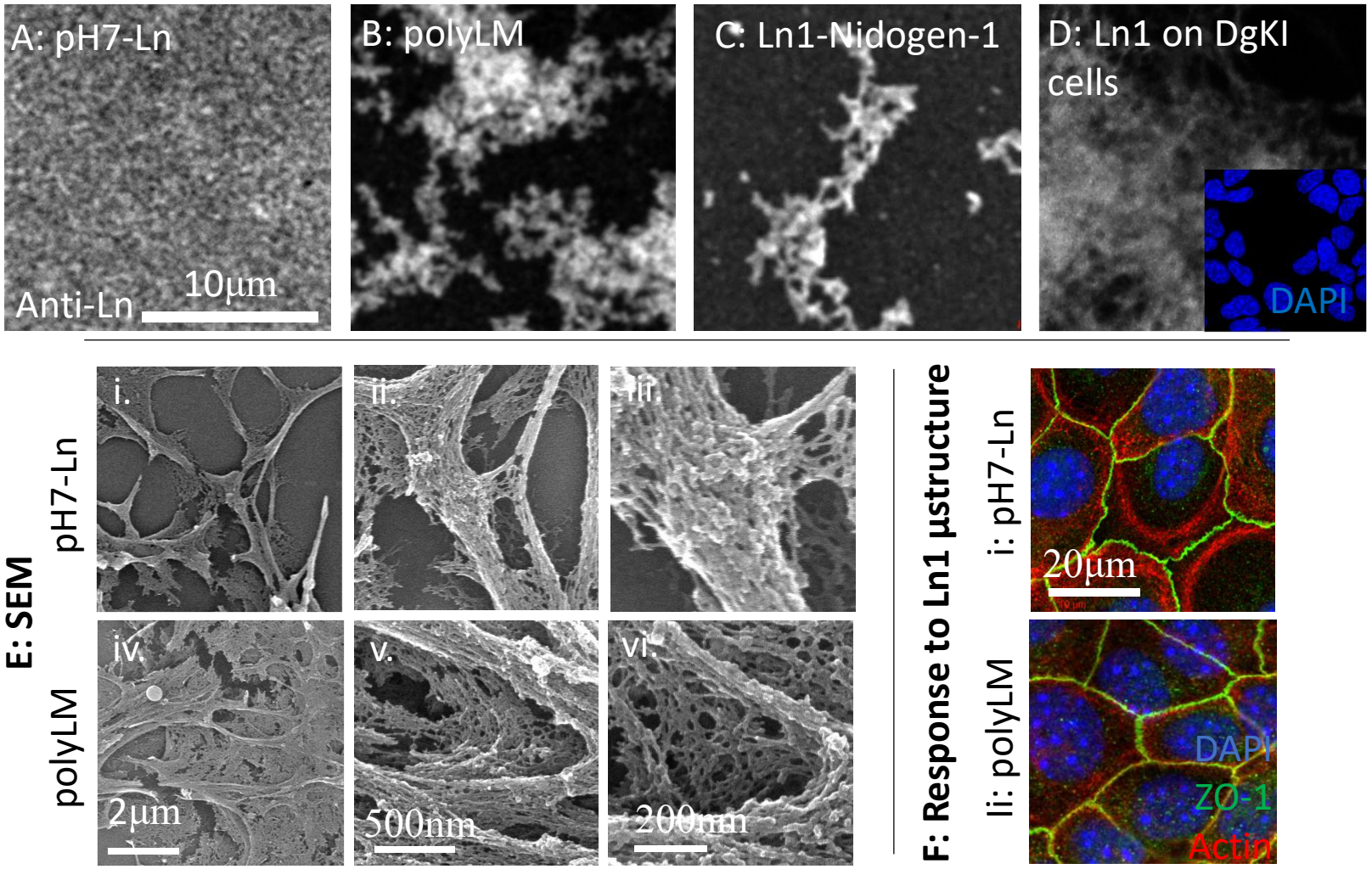

Figure 3

Click here to access/download;Figure;Fig 3.pdf

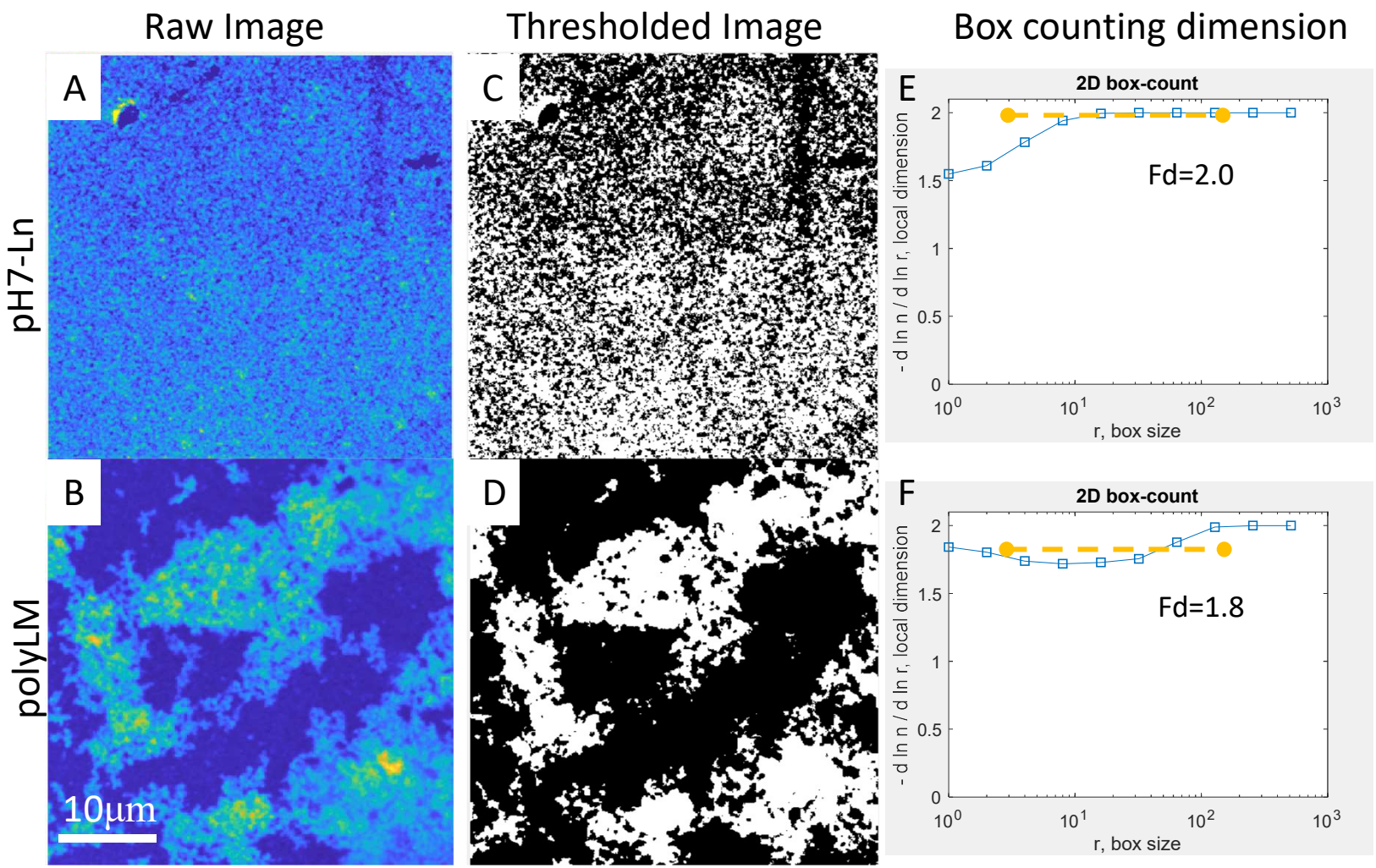

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of Material/Equipment	Company	Catalog Number
10% formalin	Sigma Aldrich	HT501128
Anti-Laminin primary antibody	Sigma Aldrich	L9393
Anti-Rabbit Alexafluor 488 secondary antibody	Thermo	A32731
Bovine Serum Albumin	Sigma Aldrich	A9418
CaCl2	Sigma Aldrich	C4901 or similar
Cell Culture Incubator	Thermo	Heracell 150 or similar
Centrifuge for microcentrifuge tubes	Eppendorf	5418 or similar
Confocal Microscope	Zeiss	LSM 710 or equivalent
Coverslips	Thermo	25CIR-1 or similar
DMEM-F12, Liquid, High Glucose, +HEPES, L-glutamine, +Phenol red	Thermo	11330107
EGF (Epidermal Growth Factor)	Sigma Aldrich	11376454001
Fluorescently Labeled Laminin	Cytoskeleton Inc	LMN02
Gentamicin	VWR	VWRV0304-10G
Glutaraldehyde	Sigma Aldrich	G5882
HCl	Sigma Aldrich	320331 or similar
Hydrocortisone	Sigma-Aldrich	H0888-5G
Insulin	Sigma Aldrich	16634-50mg
MepG Dystroglycan Knockout Cells	LBNL	N/A
NaOH	Sigma Aldrich	S8045 or similar
Normocin	Invivogen	ant-nr-1
Osmium Tetroxide	Sigma Aldrich	75633
Ovine Prolactin	Los Angeles Biomedical Research Institute	Ovine Pituitary Prolactin
Phalloidin	Thermo	A22287
Phosphate Buffered Saline	Thermo	10010023 or similar

Purified Laminin-111	Sigma Aldrich	L2020-1MG
Recombinant human Nidogen-1, carrier free	R&D systems	2570-ND-050
Sodium Acetate	Sigma Aldrich	S2889 or similar
Sodium Cacodylate	Sigma Aldrich	C0250
Sterile filters	Millipore	SLGP033RS or similar
Tris	Sigma Aldrich	252859 or similar
Triton X-100	Sigma-Aldrich	X100
Ultra-low protein binding tubes	VWR	76322-516, 76322-522 or similar
Ultrapure water	Thermo	15230253 or similar

Dear Dr. Nguyen,
Thank you for the opportunity to revise this work and the helpful comments of the editors and reviewers. We have responded to all comments using track changes as requested. We feel strongly that this has improved the quality of our work and look forward to a positive review.
Sincerely,
Claire Robertson