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Methods for the visualization and analysis of extracellular matrix protein structure and degradation

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Abstract

This chapter highlights methods for visualization and analysis of extracellular matrix (ECM) proteins, with particular emphasis on collagen type I, the most abundant protein in mammals. Protocols described range from advanced imaging of complex *in vivo* matrices to simple biochemical analysis of individual ECM proteins. The first section of this chapter describes common methods to image ECM components and includes protocols for second harmonic generation, scanning electron microscopy, and several histological methods of ECM localization and degradation analysis, including immunohistochemistry, Trichrome staining, and *in situ* zymography. The second section of this chapter details both a common transwell invasion assay and a novel live imaging method to investigate cellular behavior with respect to collagen and other ECM proteins of interest. The final section consists of common electrophoresis-based biochemical methods that are used in analysis of ECM proteins. Use of the methods described herein will enable researchers to gain a greater understanding of the role of ECM structure and degradation in development and matrix-related diseases such as cancer and connective tissue disorders.

1. INTRODUCTION

Mammalian organisms are comprised of a series of interdependent tissue compartments physically delineated by a complex mixture of proteins that comprise the extracellular matrix (ECM). In addition to providing a structural scaffold to maintain tissue architecture and topology, these proteins also define tissue specificity and serve as a depot for growth factors and other components that regulate cellular behavior within a tissue context. ECM proteins provide guidance cues to modify cell behavior based not only on biochemical stimuli but on stiffness gradients as well. This provides a cell with the ability to generate a

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distinct pattern of responses that are dependent on matrix protein composition, organization, rigidity, and topography. To this end, it is useful to incorporate methods that enable visualization of changes in matrix protein structure and organization into traditional biochemical studies of matrix degradation to enable a more comprehensive evaluation of cell:matrix interactions in the control of matrix organization and modulation of cell behavior. As collagens are the most abundant protein in the mammalian organism, comprising approximately one-third of the total body protein in humans, we have focused the methodological examples in this chapter predominantly on analysis of collagen and cell:collagen interactions.

2 ADVANCED IMAGING FOR VISUALIZATION OF COLLAGEN AND OTHER ECM PROTEINS IN TISSUES

2.1 SECOND HARMONIC GENERATION MICROSCOPY

Second harmonic generation (SHG) microscopy is a nonlinear imaging method that has been used to visualize the endogenous ECM components of various sample types in a specific and sensitive manner (see Chapter “Nonlinear optical imaging of extracellular matrix proteins” by Shih et al. in this volume for a detailed discussion of multiharmonic generation microscopy). For example, SHG can be used to image complex samples such as human and animal tissue, as well as simpler samples like collagen gels. A major advantage of SHG microscopy includes the ability to image live, unstained, and unfixed tissues as well as fixed tissue (Keikhosravi, Bredfeld, Sagar, & Eliceiri, 2014). Using ImageJ and other computer-based image analysis tools, intensities, thresholds, and forward–backward excitation measurements associated with ECM proteins can be used for quantification of SHG data (Chen, Nadiarynnkh, Plotnikov, & Campagnola, 2012).

The ease of preparing samples for SHG provides a promising means to evaluate and categorize ECM structures as potential biomarkers for disease presence and/or prognosis. For example, a tumor-associated collagen signature-3 (TACS-3) can be categorized by SHG and is associated with a poor prognosis for breast cancer survival. Knowledge of the TACS-3 has the potential to improve diagnostic capabilities (Conklin et al., 2011). Another recent application includes work using ECM properties as determined by SHG imaging as a biomarker to delineate different subtypes of ovarian cancer (Tilbury et al., 2017).

Derived from the nonlinear nature of this imaging technique, an additional value of SHG imaging is its capacity for simultaneous utilization with other imaging techniques on a given sample. This multimodal capability allows for two-photon excited fluorescence microscopy to be combined with SHG (Mohler, Millard, & Campagnola, 2003; Thomas, van Voskuilen, Gerritsen, & Sterenborg, 2014). In our laboratory, fresh peritoneal tissues containing RFP-tagged ovarian cancer cells can be imaged to visualize the interaction between tumor cells and the surrounding collagen-rich matrix. Data in Fig. 1 demonstrate that the presence of tumors in the sample is often correlated with alterations in the quaternary structure of the collagen matrix. The peritoneal collagen sample was imaged with an excitation wavelength at 860 nm and emitted light was collected at 425–465 nm. *Note that optimal excitation wavelength may vary based on laser and sample.* Based on the images shown, it is not

known whether the tumor cells are remodeling, degrading, or depositing new collagen to the peritoneal matrix. To clarify the nature of this interaction, various inhibitors could be added to the system. For example, to evaluate the contribution of enzymatic degradation to the observed tumor-related changes in matrix structure, mice could be treated intraperitoneally with matrix metalloproteinase (MMP) inhibitors prior to SHG imaging of peritoneal tissues. The coupling of SHG imaging and two-photon fluorescence microscopy has a wide range of applications beyond assessing enzymatic degradation of collagen. For example, a recent study utilized a fluorescently labeled collagen hybridizing peptide to probe mechanical damage in rat tail tendon in conjunction with SHG-detected collagen tissue architecture (Zitnay et al., 2017).

2.2 SCANNING ELECTRON MICROSCOPY

As a high-resolution complement to SHG imaging, scanning electron microscopy (SEM) can be used for visualization and analysis of the structural properties of fixed ECM proteins such as collagen (Panwar et al., 2015). An example is shown in Fig. 2 for SEM processing and imaging of peritoneal collagen. The protocol that follows details preparation of tissues, including collagen-rich tissues, for SEM imaging and was developed by the Stack laboratory in collaboration with the University of Missouri–Columbia Electron Microscopy Core and the Electron Microscopy Core at the University of Notre Dame Integrated Imaging Facility. Practical considerations for detailed protocols are provided in italics. The reader is also referred to Chapter “Ultrastructural analysis of the extracellular matrix” by Keene and Tufa in this volume for more details on SEM.

2.2.1 Tissue Preparation Protocol for SEM

1. Immediately after dissection, place tissue(s) in primary fixative of 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M Cacodylate buffer, pH 7.35 for at least 1 h at room temperature, or at 4°C overnight. *It is important to NOT let the sample(s) dry out at any time during any of the steps prior to critical point drying. The sample(s) may be stored in the primary fixative at 4°C for several weeks prior to secondary fixation. Note that primary fixative solution is very hazardous and should be used with appropriate safety measures; consult MSDS for glutaraldehyde and paraformaldehyde prior to beginning experiments.*
2. Wash sample(s) with a rotating or rocking platform 3 × 20 min in 0.1 M Cacodylate buffer, pH 7.35.
3. Place tissue(s) into the secondary fixation solution of 1% OsO₄ in 0.1 M Cacodylate buffer, pH 7.35.
4. Place sample(s) in a 100 W microwave under vacuum with dish lid (or tube caps) OFF. Set the cycle for the microwave such that it runs 1 min off, 80s on, 3 min off, 40 s on. *Note that all steps with OsO₄ must be carried out in a ventilated chemical hood for safety. OsO₄ is acutely toxic. Prevent contact with eyes, skin, and respiratory tract. Consult MSDS Information prior to working with OsO₄ and develop a Standard Operating Procedure with your institution’s safety officer before beginning experiments with OsO₄.*

5. Quickly rinse 3 × with 0.1 M Cacodylate buffer.
6. Quickly rinse once with Milli-Q water.
7. Wash 3 × with Milli-Q water for 5 min each on rocker or rotator.
8. Dehydrate the sample(s) with an ethanol gradient, microwaving at 100 W for 40 s after removal of the previous solution and addition of the new solution:
 - a. Add 20% ethanol to sample(s), microwave at 100 W for 40 s.
 - b. Add 50% ethanol to sample(s), microwave at 100 W for 40 s.
 - c. Add 70% ethanol to sample(s), microwave at 100 W for 40 s.
 - d. Add 90% ethanol to sample(s), microwave at 100 W for 40 s.
 - e. Add 100% ethanol to sample(s), microwave at 100 W for 40 s. Repeat this step for a total of three times.
9. Perform critical point drying (CPD) of the sample(s) immediately after dehydration according to manufacturer's specifications. *This is the critical step in protocol. If CPD cannot be performed immediately after dehydration, stop after step 8c and leave sample(s) in 70% ethanol.*
10. After CPD step, store sample(s) in dry 37°C incubator prior to mounting and imaging.
11. Mount dried sample(s) onto stubs. An example method of mounting a tissue specimen with silver paint is provided in the following steps.
12. Place a processed dried tissue sample on a stub covered with a carbon sticker. Apply silver paint on tissue edges with a toothpick, including up the sides of the specimen until it reaches the top surface of the tissue. *This is important in order to create conductivity between the sample tissue surface and the carbon layer. Be careful not to get silver paint on your area of interest. The tissue mounting step may be performed under a stereomicroscope to ensure correct orientation of the tissue. It is recommended that curved tissue be cut in flatter pieces, using razor blades, such that sufficient contact can be made with the carbon surface.*
13. Dry sample(s) for at least 24 h in oven at 37°C prior to imaging. Check for silver cracking prior to imaging. *If cracks are present, they need to be filled in with extra silver paint and be allowed to dry for an additional 24 h.*
14. Sputter coat sample(s) with 5–8 nm of a conductive material, such as iridium or platinum, prior to SEM imaging. *Note that thinness of sputter coat may be varied to optimize conductivity and imaging of sample(s).*
15. Store sample(s) overnight at 37°C. *Fully processed and properly stored sample(s) may be kept indefinitely for future imaging.*

2.3 HISTOLOGY

2.3.1 Immunohistochemistry—There are a variety of ways to utilize immunohistochemistry (IHC) to assess ECM proteins and degradation. IHC can be simply utilized to determine the localization of various ECM proteins in a tissue with antibodies directed against the ECM protein of interest. For example, antibodies to collagen I, collagen IV, laminin, and fibronectin have been used to demonstrate the presence and define the localization of these ECM components in the submesothelial zone of human peritoneal tissue (Witz et al., 2001). More complex approaches to utilizing IHC have been developed to assess matrix degradation. Antibodies can be produced which recognize neoepitopes present after collagen degradation by MMPs. Collagenase-catalyzed cleavage of triple helical collagen occurs at a specific site in each alpha chain located three-quarters of the distance from the amino-terminus of the protein. The new amino-termini generated on the one-quarter length (carboxyl-terminal) collagen fragment provide a de novo site for antibody recognition (Billinghurst et al., 1997). This can be used in a variety of biological applications. For example, an antibody against a neoepitope of collagen II has been used to assess the role of MMP13 in bone development (Lee, Lamplugh, Kluczyk, Leblond, & Mort, 2009).

IHC can also be utilized to visualize cellular interactions with ECM proteins. For example, to model ovarian cancer metastasis, our laboratory incubated GFP-tagged ovarian cancer cells and multicellular aggregates with mouse peritoneal tissue prior to fixation and subsequent analysis by IHC (Fig. 3). A GFP antibody was utilized to assess the location of the cells in relation to the peritoneal collagen. This method could be coupled with an appropriate neoepitope antibody as a means of assessing collagen degradation induced by MMP-expressing ovarian cancer cells. Addition of MMP inhibitors to this assay would then enable further evaluation of mechanistic details relevant to peritoneal seeding by tumor cells.

2.3.2 Trichrome Staining—Trichrome staining is used to visualize connective tissues, particularly collagen, in tissue sections. In a standard Masson's Trichrome procedure, collagen is stained blue, nuclei are stained dark brown, muscle tissue is stained red, and cytoplasm is stained pink. Fig. 4 depicts a sample Trichrome stain of formalin-fixed paraffin-embedded rabbit oral gingiva prepared via the sample protocol below.

1. Deparaffinize and rehydrate sample(s) to deionized (DI) H₂O through the steps detailed below. In between each step, drain and remove the excess of the prior solution by blotting on paper towels before going on to the next step.
 - a. Place in xylene solution for 5 min.
 - b. Place in second xylene bath for 1 min with gentle agitation.
 - c. Place in 100% ethanol for 1 min with intermittent agitation.
 - d. Place in 95% alcohol solution for 1 min.
 - e. Place in 70% alcohol solution for 1 min.
 - f. Place in H₂O for 2–3 min.
2. Bring sections to room temperature. *Do not allow them to dry.*

3. Rehydrate sample(s) in phosphate-buffered saline (PBS) for 5 min at room temperature.
4. Apply secondary fixative, Bouin's solution, for 1 h at 56°C. *Prewarm Bouin's solution to 60°C. This step may also be performed at room temperature overnight. Note that Bouin's solution is very hazardous and must be used in chemical hood. Consult MSDS prior to use.*
5. Wash slides for 1–2 min in running tap H₂O at room temperature to remove yellow color and cool the slides.
6. Briefly rinse in DI H₂O.
7. Stain with Weigert's Iron Hematoxylin (equal parts of Hematoxylin A and Hematoxylin B) for 5–10 min at room temperature. *Stain for 5 min if solution is fresh. Stain for 10 min if solution is a few days old. Weigert's Iron Hematoxylin is stable for up to 2 weeks at 4°C. Separated solutions are stable for up to 5 years.*
8. Wash under warm running tap H₂O for 10 min.
9. Rinse in DI H₂O for 1 min.
10. Stain with Biebrich Scarlet–Acid Fuchsin for 5–15 min at room temperature for cytoplasm stain of sample(s). *Biebrich Scarlet–Acid Fuchsin must be discarded after each use.*
11. Wash 3 × for 1 min with DI H₂O.
12. Place in Phosphomolybdic Acid–Phosphotungstic Acid for 10–15 min at room temperature to prepare sections to uptake collagen staining solution. *Phosphomolybdic Acid–Phosphotungstic Acid must be discarded after each use. If performing this step directly on the slides within a contained area, put Phosphomolybdic Acid–Phosphotungstic Acid solution on each sample(s) 3 × for 3–4 min at room temperature.*
13. Drain slides on tissue(s). *Do NOT rinse or dry.*
14. Stain collagen with Aniline Blue for 10 min at room temperature. *Aniline Blue solution is good for several years and may be used repeatedly.*
15. Rinse 2 × for 1 min with DI H₂O.
16. *Optional step:* Differentiate in 1% acetic acid for 3–5 min at room temperature.
17. Quickly, dehydrate with 95% and 100% ethanol with 3–4 dips per chamber. *Extended submersion in alcohol will remove Fuchsin staining.*
18. Clear in xylene with 4–6 dips per chamber.
19. Mount in a resinous medium.

3 ANALYSIS OF CELLULAR INTERACTION WITH COLLAGEN-RICH MATRICES

3.1 COLLAGEN INVASION ASSAY

In vitro analyses of matrix invasion are commonly used to assess enzymatic activity and have been widely correlated with the ability of cells to invade matrix barriers during development and disease progression. Penetration of a three-dimensional collagen-rich matrix may require proteolytic removal of physical barriers to matrix penetration. The use of proteinase inhibitors in the protocol can enable analysis of the extent to which invasive behavior is proteinase dependent. A sample protocol for invasion of three-dimensional collagen gels prepared in a Boyden chamber with a porous filter is provided below. Note that this protocol can be adjusted to accommodate the preparation and analysis of other matrix proteins such as Matrigel[©] basement membrane extract. An example showing differential invasion of Matrigel[©] and type I collagen gels by ovarian cancer cells is depicted in Fig. 5. Additionally, following the invasion assay, the ECM components can be collected and analyzed using sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with matrix protein-specific antibodies to visualize specific patterns of matrix protein degradation (Munshi & Stack, 2002).

Day 1

1. Add serum-free media (SFM) to cells overnight. *Serum contains proteinase inhibitors.*
2. Coat the underside of an 8 µm invasion chamber (BD #354578) with 500 µL of 100 µg/mL collagen I diluted in coating buffer (0.1 Na₂CO₃ at pH 9.6) at 37°C for 2h. Remove protein solution and rinse gently with coating buffer. *This step enables “invaded” cells to adhere to the bottom of the filter rather than passing through to the medium below.*
3. Coat the inside of the chambers with 100 µL of 200 µg/mL collagen I for 24 h at 4°C. *Note that collagen concentration and gel thickness may be varied according to experimental intent.*

Day 2

1. Carefully wash the chamber three times with 500 µL of SFM.
2. Trypsinize the cells and neutralize with a double amount of soybean trypsin inhibitor.
3. Spin down cells at 290 × g and resuspend in SFM at 500,000 cells/mL.
4. Add 750 µL of SFM to outer well.
5. Add 500 µL of cell suspension to inner invasion chamber. *Avoid bubbles on both sides of the filter. At this step, proteinase inhibitors can be added to the inner and outer chambers.*

6. Incubate at 37°C for 24–48 h. *The endpoint depends on the cell line and should be optimized for each individual cell line. It is recommended that an initial time course be performed to assess the optimum invasion endpoint in the 24–96 h range.*

Day 3/4

1. Use a Q-tip to remove cells from the inner chamber. *This will remove the cells from the topside of the membrane that have not migrated through the ECM and filter.*
2. Wash chamber with 1 × PBS.
3. Use Diff-Quik kit to fix and stain the invasive cells. *Dip inserts for 1 min in each solution (1—fixative, 2—staining solution I, 3—staining solution II).*
4. Wash the well in water to remove excess dye.
5. Leave to dry.
6. When dried, excise filter with scalpel.
7. Mount sample(s) on slide using Permount with cell side face down.
8. Count cells on the underside of the filter. *The suggested magnification for counting cells under a microscope is 20×. Alternatively, images can be taken and quantified with ImageJ or a similar computer-based analysis tool.*

3.2 LIVE IMAGING OF MATRIX INVASION

Live imaging of cell (or cell cluster) penetration through three-dimensional (3D) matrix constructs provides additional information relevant to analysis of mechanisms of invasion compared to the simple invasion assay protocol detailed above. Using this method of imaging allows for quantitative comparisons of fluorescently tagged cell lines under various conditions as they progress or penetrate through matrix proteins. It also allows for a more comprehensive understanding of how cell function-blocking compounds, such as MMP inhibitors, affect the penetration of the cells of interest. A sample protocol used in a recent study investigating regulation of collagen invasion by ovarian cancer cells is provided below (Klymenko, Kim, et al., 2017). Using this protocol, DOV13 ovarian cancer multicellular aggregates were found to penetrate a type I collagen matrix in an MMP-dependent manner (Fig. 6).

1. Dilute rat tail collagen type I in cell culture medium to a 1.5 mg/mL concentration. *The collagen stock MUST be kept on ice at all times to prevent solidifying. The freshly made mixture of collagen and cell culture medium MUST be used for 3D gel construction immediately, as it starts polymerizing at room temperature.*
2. Place 200 µL of fresh mixture of collagen and cell culture medium into 20mm glass wells of glass-bottomed 35mm dishes (# D35–20-1-N, In Vitro Scientific). *This 200 µL volume is defined strictly for 1.5 mg/mL collagen concentration (see step 1) and a 20mm glass area, as it provides the appropriate 3D gel height*

suitable for the capabilities of the microscope lasers used in subsequent step 5; if altering collagen concentration or dish type, the amount needed for 3D gel construction will require modification as well. This can be empirically determined based on the equipment utilized.

3. For polymerization, place the mixture in a cell culture incubator (37°C, 5% CO₂) for 20–30 min.
4. Apply 200 μL of suspended fluorescently tagged cells or multicellular aggregates, prepared per protocol above, atop polymerized 3D collagen gel constructs.
5. To image, use a confocal microscope with similar specifications to the following example: Nikon A1R-MP laser confocal microscope with ApoLWD 40 × WI lambda-S DIC N2 water-immersion objective lens for high-resolution 100 μm thick z-stack images of cell–matrix interaction. *Suggested z-stack voxel size is 0.2075 × 0.2075 × 0.6896 μm³.*
6. For matrix imaging use reflectance mode, Argonne laser with 488 nm wavelength.
7. For RFP- or GFP-tagged cell imaging use a helium–neon laser with 594 nm wavelength, or Argonne laser with 488 nm wavelength, respectively.
8. Image at time zero, and then continue imaging every 12–24 h for 7–14 days at 3–5 different ECM areas.
9. Optional: If a detailed temporal cell–matrix dynamic imaging is needed, perform continuous z-stack image acquisition from time zero up to several days using an environmental chamber at 37°C and 5% CO₂. *Note: Depending on the duration of the imaging, this might require a significant amount of a hard drive space. If desired, the acquired sequence of images can be saved as a movie during image postprocessing.*

4 ZYMOGRAPHY

4.1 IN-GEL ZYMOGRAPHY

Zymography, or substrate gel electrophoresis, is a nonquantitative method for evaluation of proteinase profiles. In this modification of SDS-PAGE, the entire gel is polymerized with a protein substrate of interest, most commonly gelatin or casein. Electrophoresis under nonreducing conditions and subsequent removal of SDS enable renaturation of the proteinase(s) in the sample, allowing for substrate degradation in the confines of the gel. This technique thereby elicits information on the estimated size of proteinases. Incubation of both sample and gel with class-specific proteinase inhibitors provides additional mechanistic depth. A sample gelatin zymography protocol is provided below and depicted in Fig. 7.

1. Incubate proteinase-containing sample(s) with nonreducing Laemmli sample dilution buffer (2.5% SDS) for 1 h on ice without boiling the sample(s). Ensure no contamination with beta-mercaptoethanol so that disulfide bonds remain

- intact. To investigate the mechanistic class of proteinase, add a class-specific proteinase inhibitor.
2. Prepare an 8%–9% SDS-polyacrylamide gel containing copolymerized gelatin or casein (0.1%). Carry out electrophoresis of sample(s). Place gel in a very clean glass container for the remaining steps; contaminants in the container may influence results.
 3. Wash gel 2 × for 20–30 min with 2.5% Triton X-100 to remove SDS and renature proteinase to restore enzymatic activity. Gently rotate at room temperature during washes.
 4. Rinse gel with water 3 × to remove Triton X-100 prior to incubation.
 5. Incubate gel for 24–48 h at 37°C in buffer of choice. For example, if analyzing MMP activity, buffer at pH 8.3 is composed of 20 mM glycine, 10 mM CaCl₂, 1 μM ZnCl₂. Note that the optimal incubation time will vary based on enzymatic activity and concentration of the proteinase-containing sample. If the concentration is low, increase incubation time.
 6. After incubation, stain gels with Coomassie blue. Regions with proteolytic activity appear as clear regions against a blue background. Note: Precautions must be taken with interpretation of results for MMP activity. MMPs are produced as propeptide-containing zymogens and propeptide removal is necessary for acquisition of proteolytic activity. However, even in the absence of propeptide cleavage, MMP zymogens gain proteolytic activity due to the presence of SDS in the gel, providing a cleared zone in the gel at the zymogen molecular weight. This form of the enzyme would not demonstrate proteolytic activity in a solution-based assay. Furthermore, overestimation of proteinase activity can occur using this method in samples containing noncovalent enzyme: inhibitor complexes such as MMP and tissue inhibitors of metalloproteinases (TIMP). MMP:TIMP complexes will dissociate during electrophoresis resulting in visualization of an active “band” on the zymogram. A solution-based assay may show significantly lower activity due to the integrity of the MMP:TIMP complex (Munshi & Stack, 2002).

4.2 IN SITU ZYMOGRAPHY

Another histological method for probing proteinase activity in tissues is in situ zymography. Frozen tissue sections can be incubated with a fluorescently labeled substrate to determine the localization of and qualitatively assess the abundance of a proteinase of interest. For example, the role of matriptase was assessed in skin cryosections from a mouse model of the connective tissue disorder Netherton syndrome using fluorescently labeled denatured casein as a substrate (Sales et al., 2010). In situ zymography can also be used to assess gelatinolytic activity as shown in Fig. 8. In this example, a section of an ovarian cancer xenograft tumor was overlaid with a quenched fluorescent gelatin (FITC-conjugated DQ-gelatin). Proteolytic cleavage of gelatin removes fluorescence quenching and enables visualization of the FITC signal. While the proteolytic activity in this sample was likely due to MMP2 and MMP9,

incubation of tissue sections with DQ-gelatin in the presence and absence of specific proteinase inhibitors can be used to provide additional mechanistic insight.

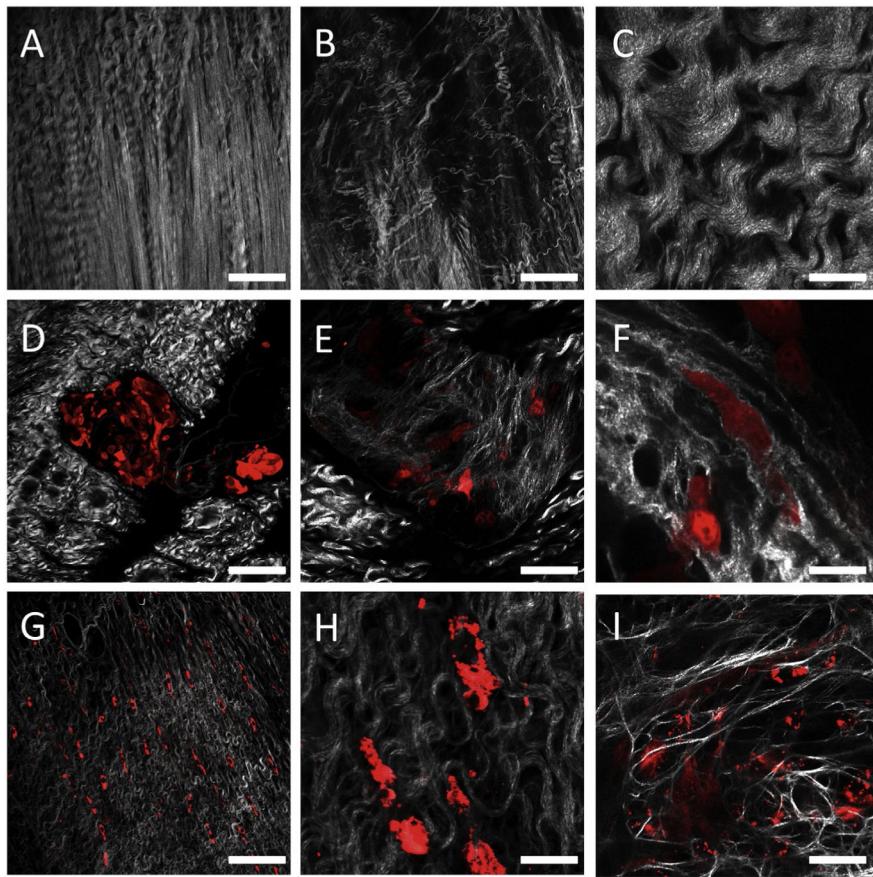
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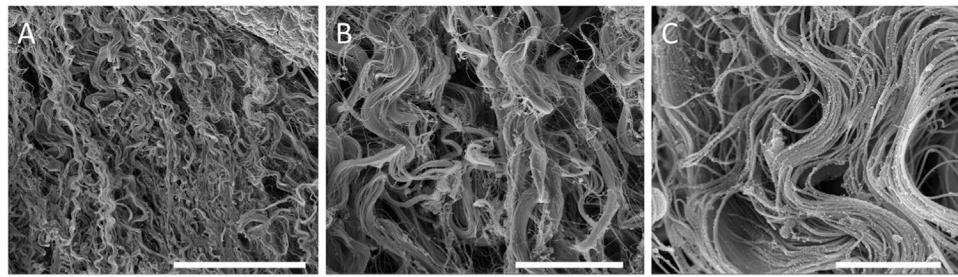
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**FIG. 1.**

Second harmonic generation microscopy (SHG) and two-photon fluorescence microscopy of mouse peritoneal tissue and intraperitoneal metastatic implants of mouse ovarian cancer tumors. (A–C) Examples of normal C57Bl/6 mouse peritoneal collagen depicting various patterns of structural organization. Gray depicts SHG collagen signal. (D–F) Examples C57Bl/6 mouse peritoneal collagen (SHG) with ID8 mouse ovarian cancer metastasis (RFP signal) (Roby et al., 2000). (G–H) Examples of omental fat band collagen (SHG signal) with FVB/NJ mouse ovarian cancer cells (RFP signal) derived from the oviductal epithelium (PTEN^{shRNA}/KRAS^{G12V}) (Eddie et al., 2015). (I) Example of connective tissue-rich tumor between the stomach and liver in the FVB/NJ PTEN^{shRNA}/KRAS^{G12V} model of intraperitoneal metastasis. Image acquisition parameters and procedures are as follows. The parietal peritoneum, omental fat band, and connective tissue tumor were placed between two cover slips (22 × 50 mm, No. 2 micro cover glass, VWR) and oriented so that when applicable, the mesothelium faced the objective (25 × XLPlan N, 1.05 NA Water) of the two-photon confocal microscope (Olympus FV1000, software FLUOVIEW FV1000). Using a Mai Tai DeepSee titanium–sapphire 690–1040 nm laser, metastatic implants and peritoneal collagen were visualized by RFP and second harmonic generation (SHG) signals, respectively. At 12% laser power, the two-photon laser was set to 860 nm and emission was simultaneously collected at 425–465 and 575–625 nm for SHG and RFP, respectively. Scale bars equal the following: A: 100 μm, B: 100 μm, C: 25 μm, D: 100 μm, E: 33.3 μm, F: 12.5 μm, G: 100 μm, H: 16.7 μm, I: 33.3 μm.

**FIG. 2.**

Scanning electron micrographs of murine peritoneal collagen. C57B1/6 murine peritoneal tissue explants were dissected with collagen layer exposed, subjected to SEM processing as described in the text, and imaged with FEI-Magellan 400 field emission microscope. Scale bars are equal to 50, 10, and 2 μm for A, B, and C, respectively.

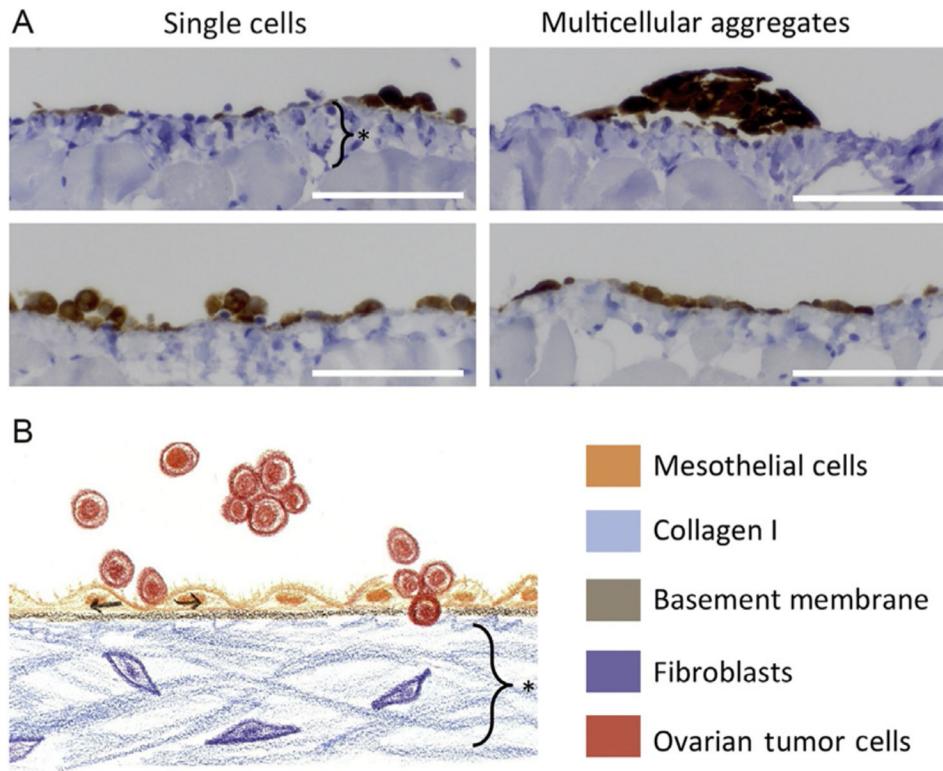
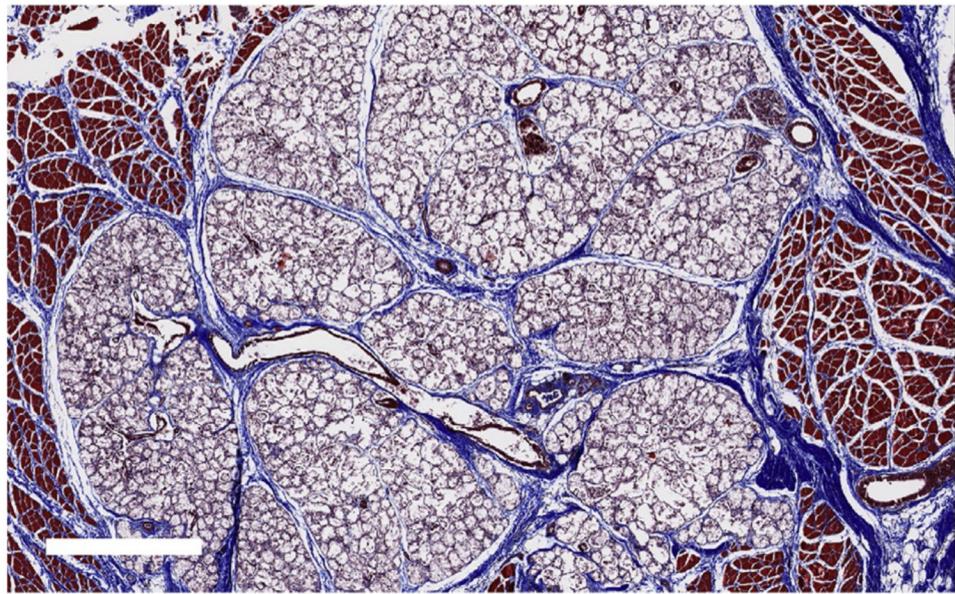
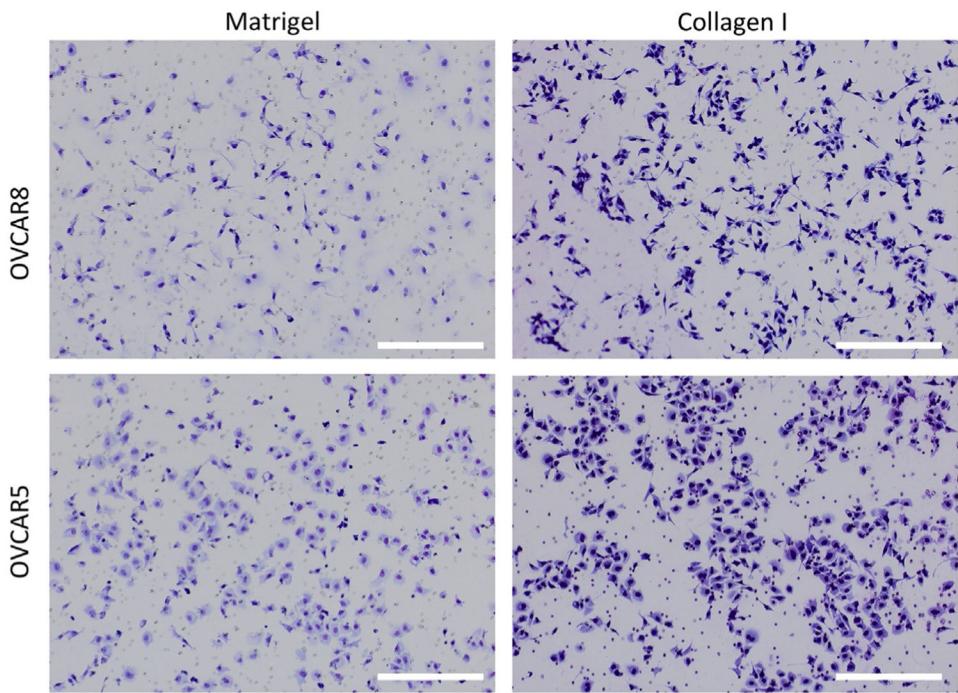


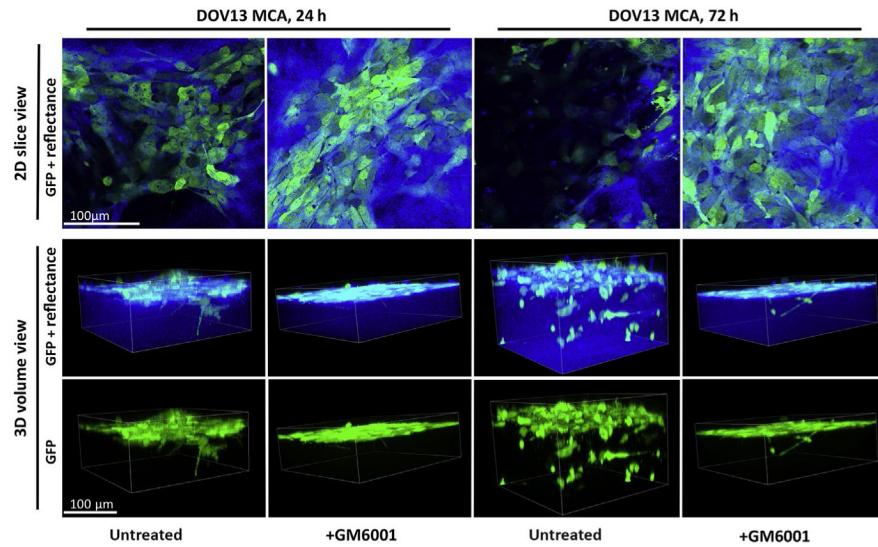
FIG. 3.
 Immunohistochemistry for GFP-tagged tumor cells in an explant ovarian cancer cell adhesion assay. (A) GFP-tagged DOV13 ovarian cancer cells or multicellular aggregates (MCAs) were incubated with a murine parietal peritoneum explant and allowed to adhere for 2h. After rinsing, samples were fixed with 10% formalin and processed for histology. IHC for GFP reveals cells and MCAs in contact with peritoneal collagen. (B) Schematic of parietal peritoneum and ovarian cancer cell adhesion is given as a reference. Asterisk indicates area of sections that consist of peritoneal collagen (predominantly type I), which is found directly under the mesothelium and is between 10 and 40 μ m thick in mice. Scale bars equal 50 μ m.

**FIG. 4.**

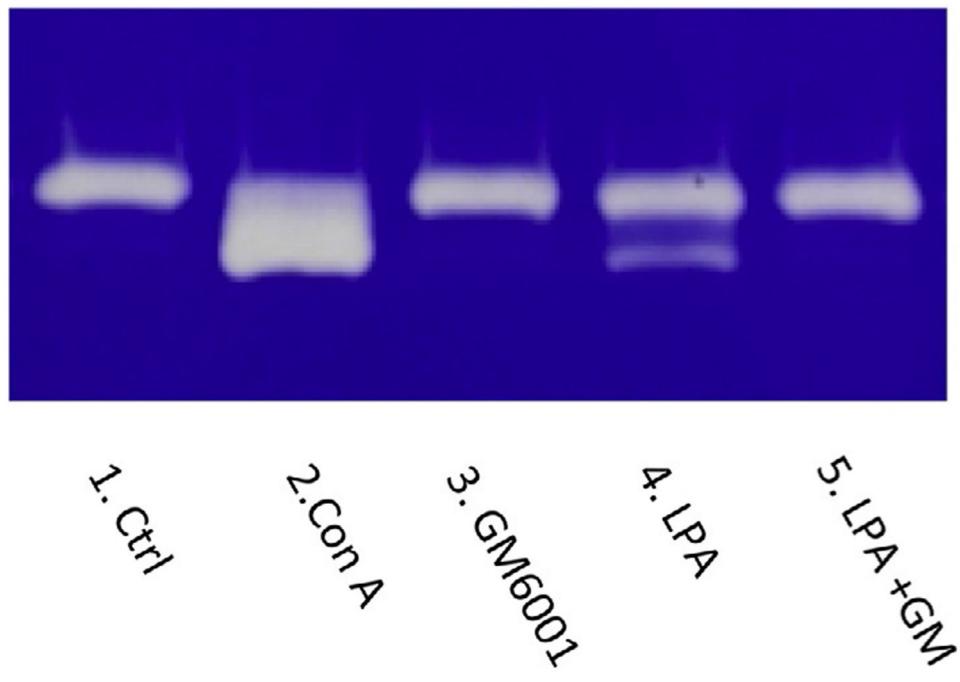
Trichrome staining of oral gingiva of rabbits. Rabbits' gingival tissues were extracted and processed using Masson's Trichrome. Collagen stains *blue* and the nuclei are stained *dark brown*. The muscle tissue is stained *red* and cytoplasm is *stained pink*. This example was prepared as detailed in the text. Scale bar is 600 μ m.

**FIG. 5.**

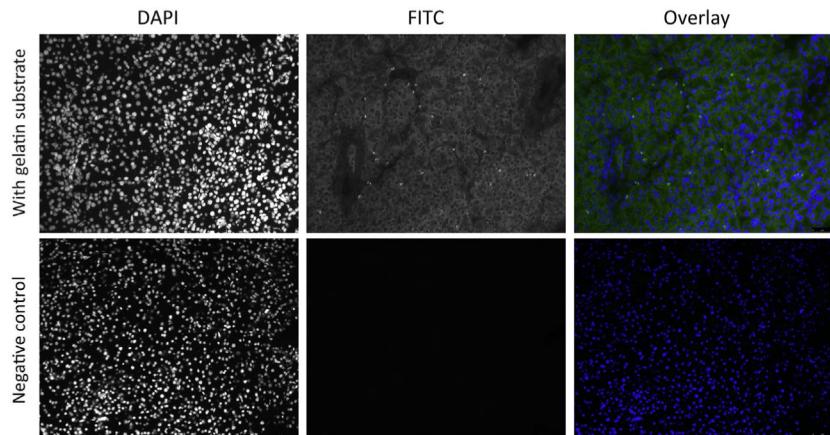
OVCAR5 and OVCAR8 ovarian cancer cell invasion of three-dimensional Matrigel[©] and collagen gels. Samples were prepared as detailed in the respective invasion assay section in the text and allowed to incubate for 24 h, after which they were imaged at 10 × magnification. Scale bars are equal to 200µm.

**FIG. 6.**

Pericellular collagen clearance and collagen invasion by ovarian cancer multicellular aggregates (MCAs). GFP-tagged DOV13 MCAs (that endogenously express the interstitial collagenase matrix metalloproteinase 14, MMP14) were prepared using the hanging drop method as described (Klymenko, Johnson, et al., 2017). MCAs were applied atop three-dimensional collagen gels (1.5mg/mL) inside a glass-bottomed dish and were incubated in the presence or absence of the broad spectrum MMP inhibitor GM6001 (25 μ M) as indicated. Nikon's A1R-MP confocal microscope was used for continuous z-stack imaging of cancer cells (*green*, fluorescence mode) and collagen (*blue*, reflectance mode) (Klymenko, Kim, et al., 2017). Representative images at 24- and 72-h incubation time points depict greater level of pericellular collagen area loss (2D slice view) and cell invasion (3D volume view) in nontreated MCAs in comparison with GM6001-treated MCAs. Scale bar: as indicated.

**FIG. 7.**

Gelatin zymography. DOV13 cells were cultured in a six-well dish to ~75% confluence and then switched to serum-free medium (SFM). Cells were then subjected to treatment as indicated below in SFM for 24 h. Conditioned medium (CM, 20 μ L) was then collected for analysis by gelatin zymography as described. Cell treatments were as follows: (1) control cells—no treatment, (2) Concanavalin A (Con A, 20 μ g/mL), (3) broad spectrum MMP inhibitor GM6001 (1 μ M), (4) lysophosphatidic acid (LPA, 80 μ M), and (5) LPA (80 μ M) and GM6001 (1 μ M). Zymography was carried out as detailed in the text. The gel was incubated in zymography buffer for 48 h in this example.

**FIG. 8.**

In situ zymography of gelatinase activity in xenograft ovarian tumor. The presence of gelatinase activity in cryosections from SKOV3 ovarian cancer xenograft tumors was assessed with in situ zymography using FITC-conjugated DQ-gelatin as described. Cleavage of the quenched fluorescent gelatin reveals the FITC signal. Tissues were snap frozen in isopentane cooled by liquid nitrogen. Original magnification of image was 200 \times .