Published in final edited form as:

Macromol Biosci. 2017 June ; 17(6): . doi:10.1002/mabi.201600478.

Imaging Cell-Matrix Interactions in Three-Dimensional Collagen Hydrogel Culture Systems

Dr Aaron R. Short^{‡,1}, Dr Catherine Czeisler^{‡,2}, Benjamin Stocker¹ [prof], Dr Sara Cole³, Dr José Javier Otero^{2,§}, and Dr Jessica O. Winter^{1,4,§}

Department of Biomedical Engineering, College of Engineering, The Ohio State University, Columbus, OH, 43210, USA

Department of Pathology and the Neurological Research Institute, College of Medicine, The Ohio State University, Columbus, OH, 43210, USA

Campus Microscopy and Imaging Facility, The Ohio State University, Columbus, OH, 43210, USA

William G. Lowrie Department of Chemical and Biomolecular Engineering, College of Engineering, The Ohio State University, Columbus, OH, 43210, USA

Abstract

Three dimensional hydrogels better replicate in vivo conditions, and yield different results from two dimensional substrates. However, imaging interactions between cells and the hydrogel microenvironment is challenging because of light diffraction and poor focal depth. Here, cryosectioning and vibrating microtomy methods and fixation protocols were compared. Collagen I/III hydrogel sections (20–100 μm) were fixed with paraformaldehyde (2–4%) and structurally evaluated. Cryosectioning damaged hydrogels, and vibrating microtomy (100 μm , 2%) yielded the best preservation of microstructure and cell integrity. These results demonstrate a potential processing method that preserves hydrogel and cell integrity, permitting imaging of cell interactions with the microenvironment.

Graphical abstract

Correspondence to: Jessica O. Winter.

^{‡,§}Authors contributed equally

Hydrogels provide more accurate representations of cell behaviors *in vivo*; however, imaging in hydrogels can be challenging. This work develops new methods that enable imaging of cellular interactions with their hydrogel microenvironments in a collagen model.

Keywords

imaging; hydrogels; immunocytochemistry; sectioning; vibratome

1. Introduction

In vivo, cells find themselves in unique three dimensional (3D) tissues with distinct physical characteristics. It has been well established that two dimensional (2D) culture methods fail to mimic many features observed *in vivo*, including topography,^[1] mechanics,^[2] and hierarchical tissue assembly,^[3] which can translate into non-physiological cell behaviors.^[4–6] Furthermore, 2D cultures cannot be used to test incisive hypotheses regarding the extent to which distinct physical forces present *in vivo* regulate cellular processes. Thus, there has been increasing interest in studying cellular interactions using three-dimensional (3D) culture systems.^[7–10] Hydrophilic polymers, especially their cross-linked forms known as hydrogels, have been extensively studied as 3D matrices because of their tunable chemistry, topography, mechanical properties, and ability to incorporate physiological cues.^[11] These matrices provide a cellular microenvironment similar to that seen *in vivo*,^[11] and have been used to investigate *in vivo* cell behaviors, including tumor morphology,^[11] phenotypic differentiation,^[12] and cell signaling,^[5] not possible using 2D culture systems.

A major limitation of hydrogel systems is the difficulty in analyzing cell behaviors *in situ*. In particular, interactions with the microenvironment that could elucidate mechanisms of cell migration, adhesion, and extracellular matrix remodeling can be difficult to evaluate. Biochemical approaches using enzymes can dissolve the hydrogel matrix (e.g., collagenase, hyaluronidase), permitting isolation of cells for the quantification of protein expression through Western blotting;^[13] however, digestion provides only limited information on cell-

matrix interactions, which are destroyed in enzymatic processing. Unfortunately, high-resolution immunocytochemistry (ICC) followed by observation using microscopy, which could yield detailed protein spatial information, is extremely challenging. Cells may reside several hundred microns below the hydrogel surface, and thick hydrogels diffract and scatter light, reducing the usable focal depth. To mitigate this, researchers can evaluate cells cultured in 3D hydrogels of reduced thickness.^[14] However, we have demonstrated that mechanical edge effects in hydrogels < 200 μm in thickness are incurred by interaction with the glass or plastic support. These effects can drastically alter cell behavior,^[15] diminishing the benefits of 3D hydrogel culture. Similarly, researchers have examined cells in a 2D configuration on the hydrogel surface. However, 2D, surface culture can yield vastly different results from 3D,^[16] limiting the effectiveness of this approach. Apart from imaging challenges, antibody penetration in thick samples is also limited and variable, and can result in weak signal or high background fluorescence.

Alternatively, specialized imaging techniques can be utilized to increase resolution. However, microscopy approaches, including confocal^[17, 18] and multiphoton microscopy,^[19] often cannot be applied to hydrogels. The thickness of the hydrogel precludes imaging at the proper focal depth because of working distance restrictions of higher magnification/N.A. objective lenses. For example, at magnifications above 60x, typical laser scanning systems have a working distance of only ~100–200 μm , well within the range in which mechanical edge effects from the support would be incurred.^[15] Thus, hydrogels studied may be 500–1000 μm thick. Often, this limitation results in the application of an optical zoom on samples imaged using lower magnification lenses that allow for deeper penetration. As images are obtained from areas of increasing depth, high degrees of light refraction typically yield extremely poor image quality (Figure 1).^[20] As an alternative, deconvolution^[21, 22] algorithms can be applied to produce a sharper image. However, this approach requires mathematical assumptions that may be inaccurate and may need to be repeated multiple times. These assumptions can lead to an increase of background noise and artifacts with each iteration.^[21]

To address these challenges, researchers have employed techniques originally developed for histological processing, such as cryosectioning. Cryosectioning has been widely used to generate hydrogel sections that permit high resolution, subcellular imaging. However, cryosectioning can also cause substantial damage to the hydrogel matrix, particularly in collagen hydrogels,^[23] and thus may not preserve cell-matrix interactions. Here, we compared two methods of creating high resolution (e.g., > 40X) images of cell-matrix interactions in collagen hydrogels using histopathological processing techniques. Specifically, we investigated hydrogel integrity following sectioning via cryosectioning and vibrating microtomy, which is commonly used in sectioning of soft tissues. Next, we evaluated the role of fixation on matrix and cell preservation; and finally, we examined the ability of these approaches to yield high resolution images of cytoskeleton, integrins, and integrin-matrix interactions (i.e., via the proximity ligation assay, PLA) in thick, 3D hydrogel cultures. Adoption of such approaches may permit cell-matrix interactions in hydrogel specimens to be investigated on standard wide-field, fluorescent microscope systems, which are more accessible to many biological researchers.

2. Experimental Section

2.1. Hydrogel Preparation

Collagen hydrogels were prepared using pre-neutralized collagen type I/III (Advanced Biomatrix, Carlsbad, CA) at 5 mg ml⁻¹, which cures at ~37 °C. Collagen was prepared at a final concentration of 2.5 mg ml⁻¹ by adding 0.5 mL of the stock solution to 0.5 mL of either phosphate buffered saline (PBS) (Fisher Scientific, Waltham, MA)(without cells) or culture media (89% DMEM F-12 (Gibco, Grand Island, NY), 10% fetal bovine serum (Quality Biologicals, Gaithersburg, MD), 1% penicillin/streptomycin (Gibco), and 1x MycoZap (Lonza, Basel, Switzerland)) (with cells). The hydrogel solution was then transferred to a 12 mm Transwell® insert (Corning, Corning, NY) in a 12 well plate and incubated at 37 °C under 5% CO₂ for 2 hours (Figure 2A). PBS (no cells) or cell culture medium (cells) was then added to completely submerge the insert and the formed hydrogel. Hydrogels were then incubated at 37 °C and 5% CO₂ for 24 hours before processing.

2.2. Hydrogel Sectioning

2.2.1. Cryosectioning—PBS was removed, and following standard ICC tissue fixation protocols developed in a clinical pathology laboratory, hydrogels in Transwell® inserts were cryoprotected using 30% sucrose solution in distilled, deionized water for 24 hours at 4 °C with rotation. The insert-mounted hydrogels were then removed and placed in a 50 ml conical tube with an intermediate freezing solution, 2-methylbutane (Sigma-Aldrich, St. Louis, MO), and placed into liquid nitrogen until frozen (approximately 2 minutes). Insert-mounted hydrogels were then removed and stored at -80 °C. The hydrogels were transferred to the cryostat, and the permeable Transwell® membrane was cut using a surgical blade, releasing the hydrogel and membrane from the insert. The hydrogel was fastened to the cryostat mount using Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Sakura, Torrance, CA). A Leica CM1510S cryostat sectioning system was utilized to create 20–50 µm sections at -24 °C.

2.2.2. Vibrating Microtome Sectioning—Following protocols developed in a clinical pathology lab and similar to those employed for sectioning brain tissue, PBS was removed, and liquid 6% (w/v), low melting point agarose (Thermo Fisher, Waltham, MA) in PBS, at ~60 °C, was poured on top of the hydrogel to completely fill the insert (Figure 2B). After the agarose cooled to ~35 °C, the hydrogel-agarose composite was carefully removed from the insert by cutting the membrane away with a surgical blade and then placed, agarose side down, into a 12-well plate (Figure 2C). Liquid 6% agarose, at ~60 °C, was poured over the composite, filling the well and encapsulating the hydrogel entirely (Figure 2D). The encapsulated hydrogel was then removed from the 12-well plate, and excess agarose was removed from the sample using a surgical blade. The hydrogel-agarose plug was then portioned into a smaller sample, ~ 5 mm in thickness, leaving in place a layer of agarose surrounding the hydrogel on four sides for support during sectioning (Figure 2E). Using Loctite super glue adhesive, samples were secured to the appropriate mounts and placed within the vibrating microtome (Lancer Vibratome Series 1000) basin filled with 1x PBS (Figure 2F). Using an optimal vibration setting of 7 and speed setting of 3, the hydrogel was

sectioned to thicknesses of ~ 100 μm . Sections were removed from the vibrating microtome with a fine paintbrush or tweezers and placed in ~3 ml of 1x PBS within a 12-well plate.

2.3. Evaluation of Hydrogel Integrity

Confocal reflectance microscopy (CRM) was employed to analyze collagen fiber formation and overall hydrogel integrity using an Olympus FV1000 microscope at an excitation wavelength of 488 nm with an attached bypass filter.

2.4. Hydrogel Fixation

Prior to sectioning, hydrogels were washed three times with 1x PBS and subsequently fixed with solutions of 2%, 3%, and 4% paraformaldehyde (PFA) in 1x PBS with 40 mg ml⁻¹ sucrose and 10 μl ml⁻¹ of 1 M NaOH. Hydrogels were completely submerged in fixative for 4 hours and then washed three times with 1x PBS for 5 minutes per wash. Finally, the hydrogel was submerged in 1x PBS and stored at 4 °C prior to sectioning.

2.5. NIH 3T3 Cell Culture

NIH 3T3 fibroblast cells, known to interact with collagen environments through their β_1 receptors,^[24, 25] were obtained from the American Type Culture Association (ATCC, CRL-1658™, Manassas, VA) and cultured in cell culture medium composed of 89% DMEM F-12 (Gibco), 10% fetal bovine serum (Quality Biologicals, Gaithersburg, MD), 1% penicillin/streptomycin (Gibco), and 1x MycoZap (Lonza) on plastic, tissue culture-treated plates. Cells were grown in a humidified incubator set to 37 °C and 5% CO₂. Feedings occurred every second or third day per ATCC recommendations, and cells were passaged weekly once the cells obtained 80–90% confluency.

2.6. Cell Encapsulation

Cells were rinsed with sterile PBS to remove excess culture medium and then detached from the cell culture surface with 0.25% trypsin (Gibco) for ~5 minutes. Cells were then pelleted by centrifugation for 3 min at 3500 rpm. Cells were re-suspended at a concentration of 400,000 cells ml⁻¹ in 2.5 mg ml⁻¹ collagen solution to a final cell concentration of 200,000 cells ml⁻¹. The hydrogel suspension was then transferred to a 12 mm Transwell® insert and incubated at 37 °C under 5% CO₂ for 2 hours. Cell culture medium was then added to completely submerge the insert and hydrogel. Hydrogels were incubated at 37 °C and 5% CO₂ for 24 hours before PFA fixation.

2.7. Immunocytochemistry (ICC) of Encapsulated Cells

Imaging of encapsulated cells was performed in intact hydrogels (control) and slices obtained using vibrating microtomy as described above. For both types of samples, PBS was removed, and materials were incubated in 250 μl of a blocking solution consisting of 5% goat serum (Fisher, ICN19135680, Pittsburgh, PA), 0.1% Triton 100x (Sigma-Aldrich, T8787, St. Louis, MO), and 95% 1x PBS for 30 minutes at room temperature. The samples were then incubated in 250 μl of primary anti-actin antibody (Sigma-Aldrich, A5441 (1:500), St. Louis, MO), in blocking solution overnight at 4 °C. Samples were then washed three times for five minutes each with 1x PBS and incubated in 250 μl of secondary antibody

(AbCam, AB97239 (1:1000), Cambridge, UK) and DAPI (slices only, Santa Cruz sc-3598 (1:1000), Dallas, TX) in blocking solution at room temperature for 1 hr in the dark. Sections were then mounted on 24×50 mm microscope slides with #1.5 glass cover slips using Prolong Gold anti-fade reagent (Molecular Probes, P36934, Grand Island, NY). Positive staining was distinguished from non-specific binding through the appropriate secondary antibody controls.

2.8. Proximity Ligation Assay (PLA)

To evaluate interactions between fibroblast $\beta 1$ integrins and collagen fibers in the hydrogel microenvironment, the PLA assay (O-link Biosciences) was employed. Samples were incubated in 250 μ l of a blocking solution consisting of 5% goat serum, 0.1% Triton 100x, and 95% 1x PBS for 30 minutes at room temperature. The samples were then incubated in 250 μ l of anti-Histone H3 (negative control) (Santa Cruz Biotechnology sc-8030 (1:200) Santa Cruz, CA), anti-collagen I (Abcam ab90395 (1:2000), Cambridge, UK) and anti- $\beta 1$ -Integrin (Abcam ab52971 (1:100), Cambridge, UK) primary antibodies in blocking solution at 4 °C overnight. Similar to standard ICC protocols, a control of only secondary antibodies was used to distinguish positive staining from non-specific binding. As an additional negative control, assays were also performed using anti- $\beta 1$ and anti-histone antibodies, as $\beta 1$ and histones are located in entirely different locations within the cell and thus not expected to interact. Samples were then processed using the Duolink PLA kit (Sigma-Aldrich DUO92101, St. Louis, MO), as per manufacturer's recommendations. In brief, samples were washed with 1x PBS three times for five minutes each, and placed in a humidity chamber consisting of a 10 cm petri dish covered by a wet towel. As per manufacturer's instructions, PLA probe mixture was added to the hydrogel samples, which were covered by parafilm to ensure even distribution over the sample surface. Humidity chambers were then wrapped in parafilm and incubated at 37 °C for 1 hour. Hydrogels were then removed from the humidity chambers, placed in a well of a 12 well plate, and washed twice for 5 minutes with the supplied Wash Buffer A on an orbital shaker. Ligase solution was pipetted onto hydrogel samples, covered with parafilm, placed in humidity chambers, and incubated at 37 °C for 30 minutes. After incubation, samples were washed with Wash Buffer A, twice for 2 minutes on an orbital shaker. Finally, amplification-polymerase solution was pipetted onto the samples, covered with parafilm, placed in the humidity chamber, and incubated at 37 °C in an incubator for 100 minutes. Samples were then washed two times for 10 minutes with the supplied Wash Buffer B on an orbital shaker and protected from light. Samples were then transferred to a microscope slide and mounted with kit provided mounting media containing DAPI.

2.9. Fluorescent Imaging for ICC Evaluation

To evaluate interactions between fibroblast Evaluation of the maximum penetration depth for imaging cells in intact hydrogels was performed using a Nikon AiR inverted, confocal microscope equipped with a 60x oil objective lens (1.4 NA, 0.13 WD). For imaging, samples were transferred to a 35mm glass bottomed dish including a #1.5 coverslip. Images were captured by adjusting the detectors to obtain optimal resolution and to maximize signal-to-noise at each imaging depth using a pixel resolution of 1024x1024. Image processing was performed uniformly using Nikon NIS elements software version 4.30.02.

Slices samples were imaged using a Zeiss LSM Axio Imager Z2 with Zen 2011 software at the following excitations: for ICC 358 nm and 493 nm, and for PLA, 358 nm and 594.

3. Results and Discussion

3.1. Effect of Sectioning Method on Hydrogel Integrity

To identify sample processing factors that influence hydrogel structural integrity crucial for imaging cell-matrix interactions, we first evaluated the effect of sectioning method on hydrogel deformation. As a model system, we investigated collagen I/III hydrogels, which display form loosely organized fibers with an intrinsic optical property that permits their visualization via light reflectance (i.e., confocal reflectance microscopy, CRM)^[26].

Previously we have shown that this approach can be used to non-invasively evaluate the structure of collagen hydrogels.^[20] Using this model, we evaluated two methods of sectioning, cryosectioning and vibrating microtomy. The paraffin wax embedding method presents several incompatibilities with hydrogels,^[23] and was therefore not evaluated. Specifically, paraffin embedding requires long dehydration processes and the application of heat. Whereas some hydrogels can be successfully processed using paraffin embedding, others can experience gross collapse and deformation (hydrogel is composed of more than 90% water^[27]).

Hydrogel integrity, representing the cellular microenvironment, was evaluated using CRM as a function of sectioning method (i.e., cryosectioning and vibrating microtomy) and sample thickness (20–100 μm). Cryosectioned hydrogels were processed at thicknesses of 20 μm and 40 μm (Figure 3) because sectioning at increased thickness (e.g., 100 μm) resulted in substantial tearing of the sample and was not feasible. Following CRM imaging, multiple areas of collagen deformation were apparent in 20 μm sections (Figure 3B vs. Figure 3A). These regions appear as black areas within the green, fibrous collagen, indicating an absence of organized fibers. Similarly, these regions were also visible in thicker, 40 μm sections, but were less pronounced (Figure 3C). In addition, the fiber distribution in both sections was noticeably irregular. We conclude that cryosectioning leads to processing artifacts in both 20 and 40 μm sections. Thus, evaluating cell-matrix interactions downstream of cryosectioning would be fraught with caveats.

We hypothesize that these artifacts most likely result from a lack of structural stability within the hydrogel during sectioning that causes sample shredding, or “chattering”. The sample preparation used in cryosectioning requires substitution of water with sucrose to reduce the size of ice crystals that form during freezing. As a result of the high water content of hydrogels, even after sucrose substitution, the hydrogel can remain brittle and can fracture during the sectioning process.^[27] In addition, the osmolarity difference between cells and the extracellular environment during sucrose addition causes dehydration of the cells.^[28] Post-processing of thawing and drying can also introduce artifacts within the sample.^[29]

We next examined vibrating microtome sectioning. Vibrating microtomes are frequently used to section brain tissue. Brain is one of the softest tissues in the body with a modulus of only a few hundred Pa,^[30, 31] similar to that of the concentrated collagen hydrogels employed here.^[32] Consistent with protocols employed for the sectioning of soft tissue,

hydrogels were first encased in 6% agarose to enhance integrity during the sectioning process. This step might not be necessary for stiffer hydrogels, such as polyethylene glycol, whose modulus can range up to MPa.^[33] Using this approach, sections as thin as 100 μm could be created. At section thicknesses $<100\text{ }\mu\text{m}$, sample integrity decreased, causing the hydrogel section to break apart. These 100 μm sections demonstrated an even distribution of collagen fibers with little to no loss of collagen structure (i.e., indicated by the absence of “dark” areas) (Figure 3D), similar to the unsectioned control (Figure 3A). These results are also consistent with previous observations of unsectioned collagen matrices.^[26, 34] We conclude that vibrating microtome sectioning can be accomplished with little disruption to the intrinsic hydrogel environment, an ideal outcome for high resolution imaging of cell-matrix interactions. Further, compared to cryosectioning, vibrating microtomy requires fewer preparation steps and can be performed using fixed or fresh tissue.

3.2. Effect of Cell Fixation Agents on Hydrogel Integrity

Next, the influence of cell preservation agents (e.g., fixatives) on hydrogel integrity was evaluated. Fixatives have the potential to influence hydrogel morphology. Standard PFA or formalin fixatives cross-link nitrogen groups via methylene bridges,^[35–37] and in collagen have been shown to form lysine-peptide linkages.^[38] To determine the potential of fixative exposure to disrupt hydrogel integrity, PFA was added at concentrations of 2–4% to 100 μm sections generated using vibrating microtomy. These concentrations were selected because the lowest reported fixative concentration that can be employed to adequately preserve multiple cellular structures is 2% PFA,^[39] whereas the concentration used in most histopathological applications for cells or tissues is 4%. Increasing fixative concentration reduced the intensity of CRM reflected light (Figure 4). We conclude that collagen fibril structures are altered by the fixation process. This most likely occurs because of cross-linking between adjacent collagen fibers.^[38] As a lower limit of 2% PFA is required for optimal cell fixation,^[40] subsequent assays were performed with 2% PFA.

3.3. Preservation of Cytoskeletal Structure in Hydrogel Sections

Next, we evaluated cell preservation; an important component of visualizing cell-matrix interactions. We examined gels sectioned using a vibrating microtome and fixed with 2% PFA. Samples obtained through cryosectioning were not examined because their poor matrix preservation (Figure 3) makes them unsuitable candidates for observing cell-matrix interactions. As a model system, NIH 3T3 mouse fibroblasts were employed. Fibroblasts are well known for their ability to remodel collagen *in vitro* and *in vivo*^[24, 25] as part of the wound healing process. Thus, fibroblasts are ideal candidates for evaluating cellular interactions with collagen hydrogels at the ultrastructural level. To evaluate cell preservation, we used ICC approaches to identify cells via their nuclei and their actin distribution. Actin rearrangement is anticipated in conjunction with collagen binding and remodeling. NIH 3T3 fibroblasts were suspended within collagen hydrogels, and sections were stained using standard ICC protocols for actin (green, Figure 5). Cell nuclei were identified using DAPI nuclear stain (blue, Figure 5). We conclude that vibratome sectioning is compatible with immunofluorescent applications requiring high magnification.

Image quality in 100 μm hydrogel sections was comparable to those created from cells cultured on cover glass, the gold standard in imaging (Figure 5), although clear differences in cellular morphology were evident, as expected. Importantly, image quality was vastly improved over that of unsectioned hydrogels not processed using this protocol (Figure 1). Cells cultured in hydrogel systems exhibited clear polarization terminating in potential filopodia as evidenced by increased actin staining, whereas cells cultured on cover glass remained rounded with lamellar sheet extensions. As expected, the formation of stress fibers is clearly visible in the cells plated on cover glass. Stress fiber formation is strongly correlated to material stiffness.^[41] In contrast, cells cultured in collagen hydrogels, which are orders of magnitude less stiff than cover glass (i.e., ~ 300 Pa vs. 100 MPa), display less organization of the actin cytoskeleton, consistent with previous observations.^[20, 42] Importantly, cells appear intact with no clear disruption in microstructure, suggesting that these methods preserve sub-cellular structure. Similar images could not be obtained in unsectioned hydrogels (Figure 1).

3.4. Imaging Cell Matrix Interactions in Hydrogel Sections

Next, we evaluated ability to image cell-matrix interactions in hydrogel sections using the proximity ligation assay (PLA). PLA is an emerging method in bioanalysis to detect interactions or co-localization of proteins in proximity of as little as ~ 16 nm.^[43] The PLA approach senses proteins via amplification of looped DNA between targets, and has previously been used in collagen systems to detect the bioconjugation of liposomes.^[44] Here, we employed PLA to evaluate the spatial distribution of collagen- β_1 binding (Figure 6), which is anticipated between β_1 integrins on the surface of NIH 3T3 cells and neighboring collagen fibers in the support matrix. It is known that β_1 integrins interact with collagen^[45] and that fibroblasts most likely remodel collagen fibers through these and similar interactions, however, little is known about the spatial relationships in these processes. In this study, we observed β_1 -collagen interactions as diffuse staining (Figure 6C) with uniform distribution. Notably, staining was not observed in the negative control (β_1 -histone interactions, Figure 6B), and staining was consistent with β_1 distribution of NIH 3T3 cells cultured inside hydrogels (Figure 6A). This suggests that the PLA assay can successfully identify β_1 -collagen interactions, and supports the premise that histological processing can be applied to 3D hydrogel-embedded cell cultures to visualize cell-matrix interactions. In addition, it should be noted that the images in Figure 6B,C have been digitally zoomed, demonstrating the ability to perform this function on samples processed with this approach.

4. Conclusions

Here, we have shown that sectioning approaches can enable imaging of cells from locations deep within 3D collagen hydrogel cell constructs. This approach obviates the need for specialized imaging techniques, such as multiphoton, confocal, or deconvolution microscopy, and can be implemented with manual fluorescence microscopes, as well as systems capable of semi-automated image acquisition and image processing. Additionally, this preparation method can be combined with advanced bioassay techniques, such as PLA. Although this work focused on fixed cells, these approaches could be equally applied to live

cells, permitting dynamic imaging. Additionally, it is likely that this approach could be expanded to additional hydrogel materials beyond collagen. Such approaches provide a unique method for *in situ* visual and quantitative analysis of interactions between encapsulated cells and their local microenvironment, which can be selectively altered by changing hydrogel composition, mechanical properties, or topography. Thus, this approach has the potential to provide new insight into molecular pathways utilized during cell adhesion and migration processes with the potential to impact many fields.

Acknowledgments

The authors kindly acknowledge support from the Metro High School Fellowship (A.R.S.) and the Pelotonia Cancer Research Fellowship (B.S.). Sections and CRM images presented in this report were generated using the instruments and services available at the Campus Microscopy and Imaging Facility at The Ohio State University. The project described was also supported by Award Number Grant 8UL1TR000090-05, from the NCATS. This work was sponsored by and represents activity of The Ohio State University Center for Regenerative Medicine and Cell Based Therapies (regenerativemedicine.osu.edu).

Appendix/Nomenclature/Abbreviations

CRM	Confocal Reflectance Microscopy
ICC	Immunocytochemistry
PFA	Paraformaldehyde
PLA	Proximity Ligation Assay

References

1. Tibbitt MW, Anseth KS. *Biotechnol. Bioeng.* 2009; 103:655. [PubMed: 19472329]
2. Pedersen JA, Swartz MA. *Annals of Biomedical Engineering.* 2005; 33:1469. [PubMed: 16341917]
3. Schindler M, Nur EKA, Ahmed I, Kamal J, Liu HY, Amor N, Ponery AS, Crockett DP, Grafe TH, Chung HY, Weik T, Jones E, Meiners S. *Cell Biochemistry and Biophysics.* 2006; 45:215. [PubMed: 16757822]
4. Weaver VM, Fischer AH, Peterson OW, Bissell MJ. *Biochemistry and Cell Biology.* 1996; 74:833. [PubMed: 9164652]
5. Cukierman E, Pankov R, Stevens DR, Yamada KM. *Science.* 2001; 294:1708. [PubMed: 11721053]
6. Griffith LG, Swartz MA. *Nature Reviews Molecular Cell Biology.* 2006; 7:211. [PubMed: 16496023]
7. Yamada KM, Cukierman E. *Cell.* 2007; 130:601. [PubMed: 17719539]
8. Schmeichel KL, Bissell MJ. *Journal of cell science.* 2003; 116:2377. [PubMed: 12766184]
9. Pampaloni F, Reynaud EG, Stelzer EHK. *Nat. Rev. Mol. Cell Biol.* 2007; 8:839. [PubMed: 17684528]
10. Lee J, Cuddihy MJ, Kotov NA. *Tissue Engineering Part B-Reviews.* 2008; 14:61. [PubMed: 18454635]
11. Weaver VM, Howlett AR, Langton-Webster B, Petersen OW, Bissell MJ. *Semin. Cancer Biol.* 1995; 6:175. [PubMed: 7495986]
12. Benya PD, Shaffer JD. *Cell.* 1982; 30:215. [PubMed: 7127471]
13. Moon JJ, Saik JE, Poche RA, Leslie-Barbick JE, Lee SH, Smith AA, Dickinson ME, West JL. *Biomaterials.* 2010; 31:3840. [PubMed: 20185173]
14. Wozniak MA, Desai R, Solski PA, Der CJ, Keely PJ. *J. Cell Biol.* 2003; 163:583. [PubMed: 14610060]

15. Rao SS, Bentil S, Dejesus J, Larison J, Hissong A, Dupaix R, Sarkar A, Winter JO. PLoS One. 2012; 7:e35852. [PubMed: 22558241]
16. von der Mark K, Park J, Bauer S, Schmuki P. Cell Tissue Res. 2010; 339:131. [PubMed: 19898872]
17. Munch JP, Candau S, Duplessix R, Picot C, Herz J, Benoit H. J. Polym. Sci. Pt. B-Polym. Phys. 1976; 14:1097.
18. Endres M, Hutmacher DW, Salgado AJ, Kaps C, Ringe J, Reis RL, Sittinger M, Brandwood A, Schantz JT. Tissue Eng. 2003; 9:689. [PubMed: 13678447]
19. Raub CB, Suresh V, Krasieva T, Lyubovitsky J, Mih JD, Putnam AJ, Tromberg BJ, George SC. Biophys. J. 2007; 92:2212. [PubMed: 17172303]
20. Rao SS, DeJesus J, Short AR, Otero JJ, Sarkar A, Winter JO. ACS Appl. Mater. Interfaces. 2013; 5:9276. [PubMed: 24010546]
21. Young IT. Methods Cell Biol. 1989; 30:1.
22. Dixon, AE., Cogswell, C. Confocal microscopy with transmitted light. Plenum Press; 233 Spring Street, New York, New York, USA; Plenum Press, London, England, UK: 1995. p. 479
23. Yang CC, Jenkins L, Burg KJL. J. Histotechnol. 2007; 30:185.
24. Ma XY, Schickel ME, Stevenson MD, Sarang-Sieminski AL, Gooch KJ, Ghadiali SN, Hart RT. Biophys. J. 2013; 104:1410. [PubMed: 23561517]
25. Tencate AR, Freeman E. Anat. Rec. 1974; 179:543. [PubMed: 4842940]
26. Friedl P, Maaser K, Klein CE, Niggemann B, Krohne G, Zanker KS. Cancer Res. 1997; 57:2061. [PubMed: 9158006]
27. Ruan JL, Tulloch NL, Muskheli V, Genova EE, Mariner PD, Anseth KS, Murry CE. Tissue engineering. Part C, Methods. 2013; 19:794. [PubMed: 23448137]
28. Ruan JL, Tulloch NL, Muskheli V, Genova EE, Mariner PD, Anseth KS, Murry CE. Tissue Eng Part C-Me. 2013; 19:794.
29. Griffiths G, McDowall A, Back R, Dubochet J. J. Ultrastruct. Res. 1984; 89:65. [PubMed: 6544882]
30. Hirakawa K HK, Hayashi T. No To Shinkei. 1981; 33:1057. [PubMed: 7317209]
31. Miller K, Chinzei K, Orssengo G, Bednarz P. J. Biomech. 2000; 33:1369. [PubMed: 10940395]
32. Helary C, Bataille I, Abed A, Illoul C, Anglo A, Louedec L, Letourneur D, Meddahi-Pelle A, Giraud-Guille MM. Biomaterials. 2010; 31:481. [PubMed: 19811818]
33. Drira Z, Yadavalli VK. J. Mech. Behav. Biomed. Mater. 2013; 18:20. [PubMed: 23237877]
34. Gunzer M, Kampgen E, Brocker EB, Zanker KS, Friedl P. Dendritic Cells in Fundamental and Clinical Immunology, Vol 3. 1997; 417:97.
35. Gustavson, KH. The Chemistry of Tanning Processes. Academic Press; New York: 1956.
36. Kiernan JA. Microscopy Today. 2000; 00-1:8.
37. Puchtler H, Meloan SN. Histochemistry. 1985; 82:201. [PubMed: 3997553]
38. Tanzer ML, Monroe D, Gross J. Biochemistry. 1966; 5:1919. [PubMed: 5963433]
39. Zeng FF, Yang W, Huang J, Chen Y. Histochem. Cell Biol. 2013; 139:735. [PubMed: 23184065]
40. Fox CH, Johnson FB, Whiting J, Roller PP. J. Histochem. Cytochem. 1985; 33:845. [PubMed: 3894502]
41. Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming WY, Weaver V, Janmey PA. Cell Motil. Cytoskelet. 2005; 60:24.
42. Buxboim A, Rajagopal K, Brown AEX, Discher DE. Journal of Physics-Condensed Matter. 2010; 22:10.
43. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gustafsdottir SM, Ostman A, Landegren U. Nat. Biotechnol. 2002; 20:473. [PubMed: 11981560]
44. Colace TV, Jobson J, Diamond SL. Bioconjug. Chem. 2011; 22:2104. [PubMed: 21902184]
45. Hay ED. Acta Anat (Basel). 1995; 154:8. [PubMed: 8714286]

Figure 1. Actin expression in NIH 3T3 cells imaged within collagen I/III hydrogels at increasing imaging depths. Images were captured at 0.2 (A), 50 (B) and 100 (C) μm from the coverslip (gel surface). ICC and confocal microscopy demonstrates a loss of resolution with increased imaging depth. Zoomed images (insets) further demonstrate loss of resolution. Scale bars = 25 μm .

Figure 2.

Process used to prepare hydrogel samples for vibrating microtome sectioning. Collagen hydrogel (pink) is cast within the Transwell insert (A). Cooled, 6% agarose (gray) is poured on top of the hydrogel, filling the well insert (B). The porous membrane, hydrogel, and agarose are removed from the insert and placed agarose side down within a well plate (C). The well is filled with cooled, 6% agarose encasing the hydrogel-agarose plug (D). The hydrogel-agarose plug is removed from the well and cut into smaller pieces as shown in (E). Smaller pieces are fixed to the vibrating microtome mount with Loctite super glue and sectioned (F): arrows represent the cutting direction.

Figure 3.

Unsectioned control hydrogel (A) and hydrogel sections generated via cryosectioning (B,C) and vibrating microtome sectioning (D) imaged using confocal reflectance microscopy (CRM). Both cryosectioned specimens (B, C) showed evidence of fiber disruption (black regions in the CRM images depicted by white arrows). These are especially evident in 20 μm sections (B), whereas thicker 40 μm sections (C) show fewer deformities, but also show areas of disruption. In contrast, 100 μm thick vibrating microtome sections (D) demonstrate good preservation of collagen fibrils within the hydrogel environment. Scale bars = 10 μm .

Figure 4.

The influence of paraformaldehyde (PFA) fixation on collagen I/III hydrogel integrity with the addition of PFA at increasing concentrations of 2%, 3%, and 4% (A-C), respectively, as observed via confocal reflectance microscopy (CRM). Collagen fibrils exhibit enhanced preservation at lower PFA concentrations. Scale bars = 10 μm.

Figure 5.

ICC and widefield fluorescence microscopy analysis of NIH 3T3 fibroblasts cultured on (A) glass coverslips and (B,C) in collagen I/III hydrogels. Cell locations are indicated by DAPI nuclear stain (blue), actin is shown in (green). NIH 3T3 cells on glass coverslips (A) clearly show actin fiber organization, including stress fibers and lamellar ruffling. Whereas images of actin distribution in cells within sectioned collagen hydrogels at low and high magnifications (B, C, respectively) demonstrate polarized morphologies with punctate staining near the cell periphery indicative of potential filopodia. Scale bars = 50 μm .

Figure 6.

Proximity ligation assay (PLA) staining for β_1 -collagen interactions (red) in NIH 3T3 fibroblasts embedded in collagen I/III hydrogels. (A) Integrin β_1 distribution shown through basic immunocytochemistry. (B) A PLA negative control showing low PLA signal from histone (not expected to co-localize with collagen) and collagen type I/III. (C) Punctate PLA staining indicates the presence of β_1 -collagen interactions evenly distributed within this microenvironment. We conclude that vibrating microtome hydrogel sections can successfully be used for advanced fluorescence bioassays. Digital zoom was applied to B, C. Scale bars = 50 μm .