Supporting Protocols

TissueSpec® ECM Hydrogel Preparation for Immunostaining

This protocol may be used prior to immunostaining cells, organoids, or patient-derived xenografts cultured in TissueSpec® ECM Hydrogels.

Introduction

Cells and organoids may form complex three-dimensional (3D) structures or exhibit tissue-specific gene expression in TissueSpec® ECM Hydrogels. Analysis of cell-surface or intracellular markers may require immunostaining of TissueSpec® ECM Hydrogels.

While TissueSpec® ECM Hydrogels are compatible with standard immunostaining techniques, additional steps may be required to optimize staining results. Notably, organized 3D cellular structures embedded in TissueSpec® ECM Hydrogels can render antigens of interest less accessible to antibodies and thus present technical challenges to immunostaining. Some formalin-fixed paraffin-embedded TissueSpec® ECM Hydrogel samples may require the use of antigen retrieval techniques prior to immunostaining.

TissueSpec® ECM Hydrogels offer cells a 3D tissue-specific microenvironment. As embedded cells may be distributed across different focal planes within thick TissueSpec® ECM Hydrogels, visualization of cells at high magnification without sectioning samples may require confocal microscopy. Autofluorescence of matrix fibers may increase background or otherwise interfere with visualization of cells within TissueSpec® ECM Hydrogels.

The following protocols may be used to prepare TissueSpec® ECM Hydrogel samples for immunostaining:

Choice of protocol depends on the desired application, antibody, imaging capabilities, and other parameters, and should be determined by the user prior to analysis of TissueSpec® ECM Hydrogel samples.

Which 'TissueSpec® ECM Hydrogel Preparation for Immunostaining' protocol should I use?

Protocol A: Immunostaining without TissueSpec® ECM Hydrogel dissociation or sectioning
In a process similar to standard 2D immunostaining protocols, TissueSpec® ECM Hydrogels are fixed, stained, and visualized without any TissueSpec® ECM Hydrogel processing. Recommended for analysis of cells cultured on or near the surface of thin (~ 100 µm) TissueSpec® ECM Hydrogels.

Protocol B: Immunostaining by dissociation of cells/organoids from TissueSpec® ECM Hydrogels
For enhanced visualization, and in cases where antibody penetration throughout the sample is insufficient, we recommend dissociation of cells/organoids from TissueSpec® ECM Hydrogels before proceeding with immunostaining.

Protocol C: Immunostaining of whole mount TissueSpec® ECM Hydrogels
For analysis and visualization of matricellular structures, we recommend mounting and histologic sectioning of TissueSpec® ECM Hydrogels before proceeding with immunostaining.

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Protocol A: Immunostaining without TissueSpec® ECM Hydrogel dissociation or sectioning

For analysis of cells cultured on or near the surface of thin (~100 µm) TissueSpec® ECM Hydrogels by immunostaining without TissueSpec® ECM Hydrogel processing.

Materials (required but not provided)

- phosphate-buffered saline (PBS) without calcium and magnesium
- 4% formaldehyde solution, neutral buffered (histology grade)

Procedure

Note: TissueSpec® ECM Hydrogels should be prepared on chamber slides or coverslips. The following procedure is intended for applications in chamber slide or 24-well plates with coverslip inserts. For suggested TissueSpec® ECM Hydrogel volumes for other multi-well formats, please refer to **Appendix A**.

Preparation of Reagents

Prepare a fresh solution of 4% formaldehyde.

Fixation of TissueSpec® ECM Hydrogels

1. Gently aspirate cell culture media while ensuring TissueSpec® ECM Hydrogel remains intact at the bottom of the well.
2. Wash TissueSpec® ECM Hydrogel samples with PBS. Gently aspirate PBS.
3. Gently add 500 µL 4% formaldehyde to fix TissueSpec® ECM Hydrogel samples.
4. Incubate at room temperature for 30 minutes. Thicker TissueSpec® ECM Hydrogels may require longer fixation times. Optimization may be required.
5. Gently aspirate 4% formaldehyde.
6. Wash TissueSpec® ECM Hydrogel samples twice with PBS.

Your TissueSpec® ECM Hydrogel samples are now ready for standard immunostaining protocols.

Troubleshooting

Immunostaining of TissueSpec® ECM Hydrogels in some applications may be difficult. In some cases, antigen retrieval may be required. We recommend subjecting samples to boiling sodium citrate (10 mM, pH 6) for 15 minutes. Other standard antigen retrieval protocols may be applicable. Optimization may be required.

Protocol B: Immunostaining by dissociation of cells/organoids from TissueSpec® ECM Hydrogels

For immunostaining of isolated cells by dissociation of cells/organoids from TissueSpec® ECM Hydrogels.

Materials (required but not provided)

- cell culture media
- Hank's Buffered Salt Solution (HBSS) with calcium and magnesium, no phenol red (Gibco® 14025092)
- Collagenase Type I (Gibco® 17100017)

Procedure

Preparation of Reagents

1. Prepare a stock solution of collagenase type I by reconstituting collagenase type I powder in HBSS at a concentration of 50 mg/mL, or according to the manufacturer's instructions.

2. Aliquot and store collagenase at –20˚C protected from light.

3. Thaw collagenase on ice prior to use. Avoid multiple freeze/thaw cycles.

4. Warm media and HBSS to room temperature prior to use.

Dissociation of TissueSpec® ECM Hydrogels

The following procedure is intended for applications in 24-well plates. Reagent volumes for other multi-well formats are provided in the **Appendix A**.

1. Culture cells or organoids in TissueSpec® ECM Hydrogel according your cell culture protocol.

   At the time of cell/organoid analysis or passaging:

2. Prepare a *working solution* of collagenase by adding 100 µL of 50 mg/mL collagenase per 1 mL cell culture media.

3. Add 300 µL collagenase-media mixture to each well of the 24-well plate containing TissueSpec® ECM Hydrogel.

   Note: collagenase-media mixture volumes should completely cover the TissueSpec® ECM Hydrogel. For suggested adjusted volumes for other multi-well formats, please refer to the **Appendix A**.

4. Incubate collagenase with TissueSpec® ECM Hydrogels at 37˚C for 30 – 60 minutes, or until TissueSpec® ECM Hydrogels are fully dissociated. Optimization may be required.

5. Transfer the dissociated contents of wells to tubes for centrifugation.

6. Gently centrifuge cells/organoids. Aspirate the supernatant.

7. Wash cells/organoids to remove any residual TissueSpec® ECM Hydrogel components or collagenase by adding 1 mL HBSS to each tube, then repeat step 6.

8. Resuspend cells in culture media and plate onto chamber slides or coverslips.

9. Incubate at 37˚C for a few hours (duration may vary with cell type) to allow for cell attachment.

10. Aspirate cell culture media, wash with HBSS, and fix cells with a fixative appropriate for immunostaining.

Troubleshooting

The dissociation of TissueSpec® ECM Hydrogels in some applications may be especially difficult. We recommend the following guidelines for optimizing dissociation of TissueSpec® ECM Hydrogels:

- Manual pipetting of TissueSpec® ECM Hydrogels to facilitate dissociation.
- Prolonging the incubation time of collagenase with TissueSpec® ECM Hydrogels in step 4.
- Following gentle centrifugation in step 6, adding fresh collagenase-media mixture and incubating fresh collagenase at 37˚C for additional time.

Protocol C: Immunostaining of whole mount TissueSpec® ECM Hydrogels

Whole mount TissueSpec® ECM Hydrogel histological immunostaining. Histological sectioning and staining can also be used to visualize cells and organoids. Preparation of TissueSpec® ECM Hydrogels for sectioning requires additional dehydration steps preceding the standard tissue preparation steps.

Materials (required but not provided)

- phosphate-buffered saline (PBS) without calcium and magnesium
- 4% formaldehyde solution, neutral buffered (histology grade)
- 100% ethanol (histology grade)
- micro-cut paraffin
- CitriSolv (or xylene, ethyl acetate, or other solvent and clearing agent)
- deionized water

Procedure

Preparation of Reagents

1. Prepare a fresh solution of 4% formaldehyde.

2. Prepare a serial dilution of histology grade ethanol in deionized water.

Fixation and dehydration of TissueSpec® ECM Hydrogels

1. Gently rinse cells/organoids in PBS.

2. Gently add an appropriate volume of 4% formaldehyde to fix TissueSpec® ECM Hydrogel samples.

3. Incubate at room temperature for 30 minutes. Thicker TissueSpec® ECM Hydrogels may require longer fixation times. Optimization may be required.

4. Wash TissueSpec® ECM Hydrogel samples twice with PBS.

5. Dehydrate samples by subsequently incubating samples for 10 minutes across increasing concentrations (v/v) of ethanol in water: 50%, 70%, 95%, 95%, 100%.

Clearing and infiltration of TissueSpec® ECM Hydrogels

1. Transfer samples to 100% CitriSolv (or other clearing agent) for 1 hour at room temperature, followed by 1 hour at 65˚C.

2. Remove samples from wells with a razor, scalpel, or other instrument.

3. Transfer samples to a mixture (1:1 by volume) of CitriSolv and micro-cut paraffin for 1 hour at 65˚C in a paraffin mold.

4. Transfer samples to 100% micro-cut paraffin for 1 hour at 65˚C.

   Note: As an alternative to paraffin embedding, samples may be frozen and sectioned and prepared for immunostaining. Following fixation, remove samples from wells with a razor, scalpel, or other instrument and embed in Optimal Cutting Temperature (OCT) compound.

This protocol may be used to dissociate TissueSpec® ECM Hydrogels for analysis or passaging of cells, organoids, or patient-derived xenografts.

The difficulty of dissociating TissueSpec® ECM Hydrogels may vary and is dependent on the type of TissueSpec® ECM Hydrogel, cells, and duration of culture. Optimization may be required for dissociation of TissueSpec® ECM Hydrogels in some applications. Please refer to the Troubleshooting section below for additional tips on how to handle TissueSpec® ECM Hydrogels that may be especially difficult to dissociate.

Appendix A

Multi-well plate	Volume
6	1500 µL
12	1000 µL
24	500 µL
48	300 µL
96	100 µL