# Protocol: Preparation of TissueSpec® hydrogels for immunostaining

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

## Introduction

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

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

TissueSpec® matrix hydrogels offer cells a 3D tissue-specific microenvironment. As embedded cells may be distributed across different focal planes within thick 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® matrix hydrogels.

The following protocols may be used to prepare TissueSpec® matrix 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 hydrogel samples.

## Which 'hydrogel preparation for immunostaining' protocol should I use?

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

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

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

## Protocol A: Immunostaining without hydrogel dissociation or sectioning

For analysis of cells cultured on or near the surface of thin (~100 µm) hydrogels by immunostaining without hydrogel processing.

## Materials

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

## Procedure

**Note:** 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 hydrogel volumes for other multi-well formats, please refer to **Appendix A** (page 6).

*Preparation of reagents*

Prepare a fresh solution of 4% formaldehyde.

*Fixation of TissueSpec® hydrogels*

1. Gently aspirate cell culture media while ensuring hydrogel remains intact at the bottom of the well.
2. Wash hydrogel samples with PBS. Gently aspirate PBS.
3. Gently add 300 µL 4% formaldehyde to fix hydrogel samples.
4. Incubate at room temperature for 30 minutes. Thicker hydrogels may require longer fixation times. Optimization may be required.
5. Gently aspirate 4% formaldehyde.
6. Wash hydrogel samples twice with PBS.

Your hydrogel samples are now ready for standard immunostaining protocols.

## Troubleshooting

Immunostaining of TissueSpec® hydrogels in some applications may be difficult. 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.

For technical support, please visit **eastriverbio.com** or email **info@eastriver.com**

## Protocol B: Immunostaining with dissociation of cells/organoids from hydrogels

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

## Materials

- Cell culture media
- Hank's Buffered Salt Solution with Calcium and Magnesium no phenol red (HBSS; Gibco 14025092)
- Collagenase I (Gibco 17100-017)

## 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® hydrogels*

The following procedure is intended for applications in 24-well plates. Reagent volumes for other multi-well formats are provided in the **Appendix** (page 6).

1. Culture cells or organoids in TissueSpec® hydrogel according to 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 hydrogel.

   Note: collagenase-media mixture volumes should completely cover the hydrogel. For suggested adjusted volumes for other multi-well formats, please refer to the **Appendix** (page 6).
4. Incubate collagenase with hydrogels at 37°C for 30 – 60 minutes, or until 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 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 to O.N. (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® hydrogels in some applications may be especially difficult. We recommend the following guidelines for optimizing dissociation of TissueSpec® hydrogels:

- Manual pipetting of hydrogels to facilitate dissociation
- Prolonging the incubation time of collagenase with 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.

For technical support, please visit **eastriverbio.com** or email **info@eastriver.com**

## Protocol C: Immunostaining with sectioning of whole mount hydrogels

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

## Materials

- 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® hydrogels*

1. Gently rinse cells/organoids in PBS.
2. Gently add an appropriate volume of 4% formaldehyde to fix hydrogel samples.
3. Incubate at room temperature for 30 minutes. Thicker hydrogels may require longer fixation times. Optimization may be required.
4. Wash 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® 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.

## Appendix A

| Multi-well plate | Volume |
|---|---|
| 96 well | 50 µL |
| 48 well | 100 µL |
| 24 well | 300 µL |
| 12 well | 500 µL |
| 6 well | 1 mL |

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