PeptiMatrix™
Next generation peptide hydrogels

STANDARD OPERATING PROCEDURE

ICC STAINING CELLS IN PEPTIMATRIX™ HYDROGELS

1. BACKGROUND

Immunocytochemistry (ICC) allows the location of specific proteins, glycans, or lipids to be visualized by fluorescence microscopy. This is accomplished by using specific antibodies that recognize the target, which can then be detected via fluorophores/other tags conjugated to the antibody. Since the antibodies are highly specific, these tags will only be visible in the presence of the molecule of interest. One of the properties of PeptiMatrix™ hydrogels is they are completely optically clear, and thus can be ideal for cell encapsulation with the end point assay being ICC staining. However, for cells grown in 3D, such as those grown in PeptiMatrix hydrogels, this may require alternative ICC protocols compared to cells grown in monolayer, to ensure appropriate penetration of the fixative, antibody solutions and other reagents.

2. RISK ASSESSMENT

Always follow your organisation's laboratory safety procedures.

Work inside an appropriate microbiology safety cabinet for your cell type. Refer to the **PeptiMatrix Safety Data Sheet (SDS)** for detailed safety, handling, storage, and first aid information relating to the hydrogel components.

If you are working with additional cell lines, media supplements, matrix additives, or other reagents, consult the relevant SDS documents for those materials as well.

3. MATERIALS

- Class II microbiology safety cabinet (or appropriate class for your cell type)
- Cells encapsulated in PeptiMatrix hydrogels, plated in appropriate microplate e.g., black-bordered well plate with clear optical bottom (e.g. ThermoFisher #165305 - Nunc™ 96-Well Optical-Bottom Microplate, black, TC surface)
- P1000, P200, P10 pipettes and filter tips

- Dulbecco's Phosphate Buffered Saline (DPBS)
- 4% Paraformaldehyde
- Triton X-100 **or** Tween-20
- Bovine Serum Albumin powder
- Appropriate primary and secondary antibodies
- Foil
- Parafilm

Optional materials:

- Rhodamine phalloidin (e.g., ThermoFisher #R415, 300 units of lyophilised product, reconstituted in 150 µL of anhydrous DMSO to yield a 400X stock solution of 66 µM)
- DAPI (300 µM stock concentration)
- Scalpel (optional for hydrogels in transwells)
- Anti-fade mountant with DAPI or DAPI solution (optional for hydrogels in transwells)
- Glass coverslips (optional for hydrogels in transwells)
- Nail varnish (optional for hydrogels in transwells)

4. METHODS

4.1 Fixation of cells in hydrogels

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size you are using.

For reference, the steps below describe the process using cells encapsulated in **100 µL of hydrogel** per well plated into a **96-well plate**.

1. In a Class II MSC, using a P200 pipette, aspirate the old media on top of the gel from the well, removing **~75%** of the liquid volume to minimise risk of disturbing the gel.
2. Rinse wells with 100 µL DPBS for 5 mins before removing the liquid volume as described above in Step 1.
    - Depending on the stability of the gels, you may wish to wash them **once or twice** (PMCORE100 will be the least stable, PMCORE150 will be the most stable).
3. Add 100 µL 4% Paraformaldehyde to each well and incubate for 60 minutes at room temperature.
4. Remove the liquid volume as described in Step 1. Rinse wells with 100 µL DPBS for 5 mins before removing the liquid volume as described above.
    - Depending on the stability of the gels, you may wish to wash them **once or twice** (PMCORE100 will be the least stable, PMCORE150 will be the most stable).
5. Add 100 µL DPBS to each well and store the plate in the fridge until carrying out the staining procedure (optional step, can go straight onto staining procedure).

- It is recommended to stain the plate **within 1 week** of fixing, as longer storage times may affect gel stability.

4.2 Standard staining procedure of cells in hydrogels

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size you are using.

For reference, the steps below describe the process using cells encapsulated in **100 µL of hydrogel** per well plated into a **96-well plate**.

1. Using a P200 pipette, aspirate DPBS on top of the gel from the well, removing **~75%** of the liquid volume to minimise risk of disturbing the gel.
2. Add 100 µL blocking buffer per well.
    - The most commonly used blocking buffer with PeptiMatrix hydrogels is 0.5% BSA PBST (PBS + 0.1% Triton X-100), but consider the optimal conditions recommended for the chosen secondary antibody e.g. if it raised in goat, it may be recommended to use 10% goat serum etc.
3. Incubate the plate for 30-60 minutes at room temperature to block nonspecific antibody binding.
4. Aspirate the liquid volume as described in Step 1.
5. Prepare primary antibody staining solution in blocking buffer.
    - Allow 100 µL per well plus an extra 10% to allow for pipetting error.
    - When selecting the antibody concentration, consider that the gels are almost entirely water and will therefore dilute your antibody. If staining 100 µL gel with 100 µL antibody staining solution, the antibody concentration should be doubled so that when added to the gel, it matches the desired final concentration.
6. Incubate plate with primary antibody(s) and any antibody isotype controls diluted in blocking buffer, plus a no primary antibody control at 4 °C overnight.
    - It is recommended to cover plate in Parafilm to avoid evaporation.
7. Aspirate the liquid volume as described in Step 1. Rinse wells with 100 µL DPBS for 5 mins before removing the liquid volume as described above.
    - Depending on the stability of the gels, you may wish to wash them **2-3 times** (PMCORE100 will be the least stable, PMCORE150 will be the most stable).
8. Prepare secondary antibody staining solution in blocking buffer.
    - Allow 100 µL per well plus an extra 10% to allow for pipetting error.
    - When selecting the antibody concentration, consider that the gels are almost entirely water and will therefore dilute your antibody. If staining 100 µL gel with 100 µL antibody staining solution, the antibody concentration should be doubled so that when added to the gel, it matches the desired final concentration.

- If phalloidin stain is being used, this can also be added at a final concentration (after being diluted in the gel) of 1:500.
- If DAPI stain is being used, this can also be added at a final concentration (after being diluted in the gel) of 1:500.

9. Incubate plate with secondary antibody(s) and any controls at 4 °C overnight.
    - It is recommended to cover plate in Parafilm to avoid evaporation.
10. Aspirate the liquid volume as described in Step 1. Rinse wells with 100 µL DPBS for 5 mins before removing the liquid volume as described above.
    - Depending on the stability of the gels, you may wish to wash them **2-3 times** (PMCORE100 will be the least stable, PMCORE150 will be the most stable).
11. Add 100 µL DPBS to each well, then image plate as soon as possible on a fluorescent/confocal microscope.

4.3 Standard staining procedure of cells in hydrogels in transwells

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size/transwell size you are using.

For reference, the steps below describe the process for cells encapsulated in 200 µL hydrogel plated into transwell inserts suitable for 24 well plates.

1. Carry out fixation Steps 1-5 as described in Methods section 4.1, scaling up the reagent volumes accordingly and with the changes described below:
    - Remove media from **both** the well (basal) and insert (apical) chambers.
    - Split the liquid volume of 4% Paraformaldehyde between **both** the well (basal) and insert (apical) chambers to ensure complete fixation (e.g. 1 mL split 50:50 in the apical and basal chambers of a 24 well plate transwell insert).
2. Once fixed and washed, remove the transwell from the 24 well plate using tweezers and remove the membrane from the bottom of the transwell using a scalpel.
    - Run the scalpel around the edge of the transwell and remove the membrane using tweezers.
3. Place the transwell on desired staining container e.g. an empty well plate, a coverslip or imaging dish (consider the size to conserve staining solution). Allow the hydrogel to attach to the glass/plastic. Slide the transwell up carefully, leaving behind the hydrogel attached to the surface. Alternatively, gently push the gel out of the transwell using the wide end of a filter tip.
4. Carry out staining procedure Steps 2-10 as described in Methods section 4.2, scaling up the reagent volumes accordingly.
5. Transfer stained hydrogel carefully to a glass coverslip or glass-bottom well plate if desired.

- If the gel integrity is low (PMCORE100), it can be transferred to a glass slide, excess DPBS removed, and sealed with a coverslip and nail polish to stain as a monolayer if desired.

6. Visualise stained hydrogel as soon as possible on a fluorescent/confocal microscope.

5. DISPOSAL

Dispose of hydrogels containing cells, media, or matrix components according to your local guidelines for biological waste.

Dispose of glass and contaminated sharps in appropriate bins.

Dispose of any Triton X-100, Tween-20 and Paraformaldehyde in separate waste pots and refer to institute guidelines for safe disposal.

6. DOCUMENT HISTORY

Version	Date	Summary of Changes
1.0	**05 Feb 26**	First version of customer facing SOP, adapted from internal PeptiMatrix ICC procedures.