SOP TITLE:	VERSION:	
PM-PD-SOP3.1 Staining cells in PeptiMatrix hydrogels using ICC	1.3	
AUTHOR & RESEARCH TEAM: Johnathan Curd (JWC) - Product Development	STATUS: Shared	

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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. 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

Please refer to PM-RA0 for a full risk assessment of the following protocol. Ensure that you fully understand the potential risks and have received the appropriate training before starting any work.

3. MATERIALS

- Class II Microbiological Safety Cabinet (or appropriate class for your cells/materials)
- Cells in gels for staining
- Parafilm
- Masking tape
- Scalpel blade
- 24 well plate
- P1000, P200, P20 filter tips
- P1000, P200, P20 pipette
- Dulbecco's Phosphate Buffered Saline (PBS)
- 4% paraformaldehyde
- Triton X 100 **OR** Tween 20
- Bovine Serum Albumin (BSA)
- Primary and secondary (and possibly tertiary) antibodies as required for staining (refer to hazard sheet for each

individual antibody)
- Phalloidin
- Foil
- Prolong gold/diamond anti-fade mountant with DAPI, or DAPI solution
- Glass coverslips
- Nail varnish

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Product	Supplier	Cat No	Storage
DPBS	Gibco	14190-094	RT
Paraformaldehyde (Diluted to 4% with DPBS)	Polysciences	18814	4% solution at 4 °C; 16% stock in chemicals cupboard
Triton X-100	Sigma	X100	Chemicals cupboard (RT)
Tween 20	Sigma	P1379	Chemicals shelf (RT)
Bovine Serum Albumin	Sigma	5482	4 °C
Phalloidin	Life Technologies	F432/R415	-20 °C
DAPI solution	Invitrogen	D3571	300 µM working solution at 4 °C; stock solution at -20 °C

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4. METHODS

Fixation

1. In a Class II MSC, remove medium from the gels for staining, and rinse with PBS. If your gels are in well plate inserts, take great care removing/adding medium/PBS from the inside of the insert. Depending on the stability of the gels, you may wish to wash them once or twice (5 min per wash).
2. Cover with the minimum amount of 4% paraformaldehyde (we use 16% diluted down to 4% with DPBS): 100 µL per 96-well or 1 mL per 24-well, split between the well itself and the insert, is sufficient.
3. Cover, and allow to fix for 60 minutes at room temperature.

4. Wash once or twice (depending on gel stability) with PBS (5 min per wash). You can store your samples in DPBS in the fridge for up to one week at this point, sealing them well with parafilm.

Standard Staining Procedure

1. Remove PBS.
2. For gels grown in inserts, use a scalpel to score around the rim of the membrane at the insert base. Remove and discard the membrane, using forceps if necessary. Carefully transfer to a 24-well plate well, or alternatively to an 1 mL Eppendorf tube or 96-well plate well if your gel will fit comfortably (this helps to conserve staining solutions).
3. Cover with blocking buffer: a simple starting point is 0.5% BSA PBST (PBS 0.1% Triton), but consider that if your secondary antibody is raised in goat then it can be useful to also use 10% goat serum etc.
4. Incubate cells with block for 30-60 minutes at RT to block nonspecific antibody binding.
5. Remove block.
6. Incubate cells with primary antibody(s) (diluted in blocking buffer) overnight at 4 °C. Cover plate with parafilm. (No antibody and antibody isotype controls can also be added at this point). Make up 10% extra to allow for pipetting error. 100 µL per 96-well or 200 µL per 24-well is sufficient.
    1. When selecting which antibody concentration to use, consider that the gels are mostly made up of water and are therefore going to dilute your antibody as well. For example, if staining 100 µL of gel with 100 µL of staining solution, you should double the concentration of your staining solution so that when diluted by the gel it matches your desired final concentration.
7. Remove solution and, depending on the stability of the gels, you may wish to wash them two or three times (5 min per wash).
8. Incubate cells with secondary antibody(s) (diluted in blocking buffer) as appropriate for the species/isotype used in step 6, overnight at 4 °C.
    1. If phalloidin staining is being used, this can also be added at this point at a final concentration (after being diluted by the gels) of 1:500.
    2. If DAPI staining is being used, this can also be added at this point at a final concentration (after being diluted by the gels) of 1:500.
9. Optional tertiary antibody step here if needed.
10. Remove solution and, depending on the stability of the gels, you may wish to wash them two or three times in DPBS (5 min per wash).
11. Transfer to a glass coverslip or glass- bottom well plate if desired (if the gel integrity is very low, it can be transferred to a glass slide, excess PBS removed, and sealed with a coverslip and nail polish as for staining a monolayer).
12. View as soon as possible on a fluorescent/confocal microscope.

5. DISPOSAL

1. Dispose of cell culture medium by pouring into waste pot/aspirating into vacuum trap containing Chemgene. Leave in Chemgene for at least one hour.
2. Dispose of any unneeded glass in appropriate glass bins.
3. Dispose of contaminated sharps in appropriate sharps bins.
4. Dispose of any undiluted Triton X-100 or Tween 20 in separate waste pots. All diluted reagents are not considered

to be hazardous and may be disposed of directly.

User Initials Legend

JWC Johnathan Curd
MES Mikayla Shelton