Preparation of Cell-Mate3D™ Cocoon for Scanning Electron Microscopy

Note: The purpose of this protocol is to serve as a guide for preparation and imaging of Cell-Mate3D™ via Scanning Electron Microscopy (SEM). Please consult your SEM operator for specific techniques related to preparation and handling of samples, reagents, and usage of the Scanning Electron Microscope.

Preparation of Fixative

Fixative consists of: 3% paraformaldehyde, 1.5% glutaraldhyde, 2.5% sucrose, 5mM $CaCl_2$, 5mM $MgCl_2$ in a 100 mM Na cacodylate buffer, ph 7.4

For example, to make 100mls:

- 3 g paraformaldehyde prill (EMS #19200)
- 2.5 g sucrose
- add 30 mL ddH_20
- add 8-10 drops 1 N NaOH

Under the fume hood: Heat and stir at medium speed to > 50°C. Be careful. ***Do not let it reach 60 degrees C***. If it does not dissolve, add 2 drops of 1 N NaOH.

When fully dissolved, remove from heat for a minute or so. Combine with (**in order**):

- 50 ml 0.2M Na Cacodylate (EMS #12300)
- 1 mL $CaCl_2$ (0.5M stock)
- 0.5mL $MgCl_2$ (1M stock)
- 15 mL glutaraldehyde (10% stock in ampules). (EMS #16120).
- add ddH20 to bring up to 100 mL
- Check pH with pH paper to ensure pH of 7.4

Aliquot unneeded fixative and freeze at -20°C. Leave extra space in the aliquot tubes as the fixative will expand as it freezes.

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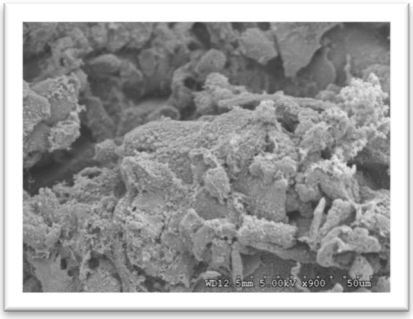
Protocol:

1) Fix samples in a solution of 3% paraformaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose in 0.1 M sodium cacodylate buffer containing 5mM $CaCl_2$ and 5mM $MgCl_2$ (pH 7.4) overnight at 4°C (see "preparation of fixative" above)
2) Rinse three times in 0.1 M sodium cacodylate buffer containing 5mM $CaCl_2$ and 5mM $MgCl_2$ (pH 7.4)
3) Place in 1% osmium tetroxide in 0.1M sodium cacodylate buffer containing 5mM $CaCl_2$ and 5mM $MgCl_2$ (pH 7.4) overnight at 4°C
4) Rinse samples in ultrapure water 3 times, for 10 min each time
5) Dehydrate in a graded series of ethanol solutions as follows:
    a. 25% twice, 5min each
    b. 50% twice, 5min each
    c. 75% twice, 5min each
    d. 95% twice, 5min each
    e. 100% three times, 5min each
6) Process samples in a critical point dryer with samples mounted onto metal stubs with double-sided carbon adhesive tabs
7) Sputter coat with gold-palladium
8) Examine with scanning electron microscope at an accelerating voltage of 5 kV



SEM image of hMSCs in Cell-Mate3D™

References:

- Perkins EM and McCaffery JM. Conventional and immunoelectron microscopy of mitochondria. Methods Mol Biol 2007; 372 467-83. pmid:18314746.

Safety Disclaimer:

Only competent and trained personnel using appropriate personal protective equipment and working within a controlled environment should handle all chemicals and perform the protocol described herein. Prior to performing this protocol, users should review appropriate safety information, including the manufacturers MSDS, related to the components used in this protocol. Bioactive Regenerative Therapeutics, Inc. shall not be held liable for any loss, injury or damage as a result from the use of this protocol.