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1. Culture 3D spheroids in suspension according to application-specific recommendations.
2. Transfer the spheroids to a 1.5-ml microcentrifuge tube and centrifuge at 800 rcf for 3 min. Aspirate media.
 - *Total cell numbers should be large enough to yield a visible spheroid pellet upon centrifugation (~1e6 cells or greater)*
3. Wash cells in 1 ml ice-cold PBS and spin at 800 rcf for 3 min.
4. Aspirate the supernatant and fix the spheroids by resuspending in 0.5 ml freshly prepared 3.7% PFA. Incubate for 15 min at room temperature.
 - *Stock solutions of 37% PFA should be prepared according to Wang et al. Meth. Enzymol. 85:514 (1982) and stored in single-use aliquots at -20°C*
5. Spin at 800 rcf for 3 min. Discard supernatant.
 - *Fix samples in a fume hood and dispose of PFA as hazardous waste*
6. Wash spheroids three times in 1 ml PBS at room temperature. Spin at 800 rcf for 3 min and discard supernatant between washes.
7. Incubate spheroids in 0.5 ml 15% (w/v in PBS) sucrose for 15 min, followed by 0.5 ml 30% (w/v in PBS) sucrose for 15 min.
 - *Sucrose incubations dramatically improve the morphology of the spheroids after freezing and sectioning*
 - *Spin at 800 rcf for 3 min and aspirate the sucrose solution between steps*
8. While the samples are equilibrating in the sucrose solutions, cover the base of small cryomolds (VWR #25608-922) with ~1 mm thickness of clear Neg-50 embedding medium (VWR #84000-154).
9. After the embedding medium has settled uniformly at the base of the cryomold, snap freeze the cryomold in a dry ice-isopentane bath. Keep the frozen cryomolds on dry ice.
10. Remove the residual 30% sucrose solution from the microcentrifuge, resuspend the spheroids in 0.2 ml yellow Neg-50 embedding medium (Fisher #22050454), and transfer carefully to the center of the frozen white Neg-50 embedding medium of the cryomold.
 - *The fellow tint provides contrast for the cell material on a white background when cryosectioning, and it does not cause fluorescence artifacts in the FITC, rhodamine, or Cy5 fluorescence channels*
 - *Use a P200 micropipette with a cut tip and avoid making bubbles when resuspending; do not touch the frozen Neg-50 when dispensing*
11. Fill the remainder of the cryomold with clear Neg-50 and snap freeze the cryomold in a dry ice-isopentane bath. Keep the embedded spheroids on dry ice and embed the remaining samples.
12. Wrap the embedded samples in tinfoil and store at -80°C for 6–12 months or more. Isopentane can be stored at room temperature and reused indefinitely (it should not be disposed of down the sink).