Clearing Spheroids with Corning® 3D Clear Tissue Clearing Reagent in Corning Spheroid Microplates

Guidelines for Use

The opacity of a three-dimensional (3D) spheroid is a barrier to imaging the entirety of the spheroid structure. Light scattering limits imaging to the outermost cells, thus introducing a sampling bias in imaging analysis. To thoroughly characterize a spheroid, it is necessary to query the entire structure, which necessitates optical tissue clearing. Corning 3D Clear tissue clearing reagent is specifically designed for clearing of spheroids and other 3D cell cultures.[1]

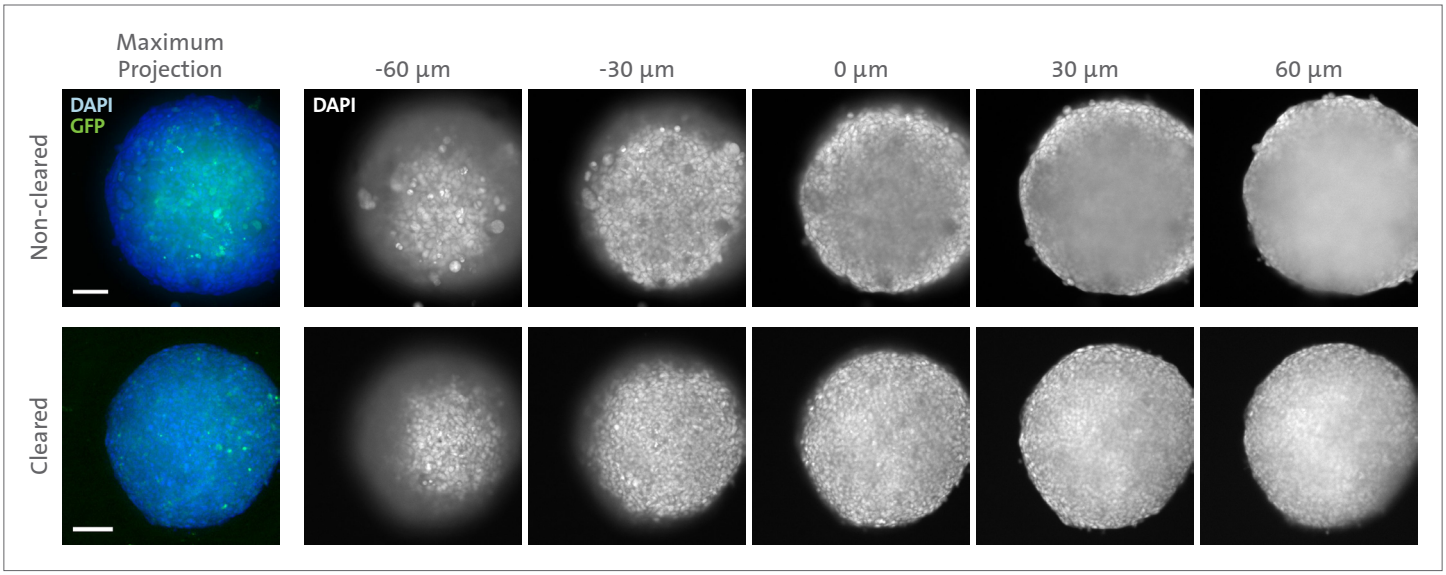
3D Spheroid Cultures

The following protocol is intended for processing of spheroids (<500 µm diameter) that have been cultured in the Corning 96-well spheroid microplate (Corning 4515 and 4520) and express fluorescent protein(s). More information on generating 3D cell culture models in the spheroid microplate can be found in the library of Corning technical literature[2-6]. With optimization, this general protocol can be adapted and scaled for 3D cell cultures generated in numerous formats including the 384- and 1536-well spheroid microplates, Corning Elplasia® plates, and Ultra-Low Attachment (ULA) surface-coated vessels.

1. Gently wash wells 2X with Ca^{2+}/Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS; Corning 21-031-CM) with 200 µL per well per wash step.

NOTE: For each solution exchange, carefully aspirate solution from each well leaving ≥50 µL remaining to avoid disturbing the spheroids. A pin tool or liquid handler is recommended for solution aspiration. Solution can be added manually to each well via a multi-channel pipettor or automatically via a liquid handler. If necessary, microplates can be centrifuged 1 to 2 minutes at 200 x g to settle spheroids after solution exchange

2. Fix spheroids in 10% neutral buffered formalin (200 µL/well) for 15 to 30 minutes at room temperature with gentle agitation on orbital shaker.
3. After fixation, gently wash wells 2X with DPBS with 200 µL/well per wash step. At this point, microplates can be sealed and stored at 4°C for up to 3 days.



Tissue clearing with Corning 3D Clear reagent enables visualization of the entire 3D spheroid structure. HT-29/GFP cells were seeded at 2 x 10^4 cells/well and cultured for 72 hours. Spheroids were processed according to the protocol listed and imaged on a Thermo Fisher CellInsight™ CX7 High-Content Screening Platform using a 10X objective. Representative maximum projection composite images of the GFP and DAPI signal (left) and single DAPI images acquired at increasing imaging depths (right). Scale bar = 100 µm.

NOTE: The subsequent steps are indicated for nuclear staining of spheroids expressing fluorescent protein. For permeabilization, immunolabeling, and clearing of 3D cell cultures, see previously published protocols.[7-9]

4. Stain spheroids with DAPI (200 µL/well) at a final concentration of 1 µg/mL for 15 minutes at room temperature in the dark with gentle agitation.
5. Gently wash wells 2X with DPBS with 200 µL/well per wash step.
6. Dehydrate spheroids with sequential 15-minute ethanol washes (200 µL/well) at 4°C with gentle agitation.
   - 30% Ethanol in DPBS
   - 50% Ethanol in DPBS
   - 70% Ethanol in deionized water (Corning 25-055-CI)
   - 90% Ethanol in deionized water
   - 100% Ethanol
7. Following the final dehydration step, aspirate as much ethanol as possible without disturbing the spheroids. Add Corning 3D Clear reagent (Corning 5733) to each well (200 µL/well) and clear spheroids for 15 minutes at room temperature in the dark with gentle agitation.

NOTE: Extend the incubation with Corning 3D Clear reagent by 30% to 50% if necessary for large, dense spheroids (<500 µm diameter). Corning 3D Clear reagent is intended for 3D cell cultures up to 500 µm in thickness.

8. Image spheroids in Corning 3D Clear reagent directly in the spheroid microplate. Alternatively, microplates can be sealed and stored at 4°C in the dark indefinitely for imaging at a later time, though it may be necessary to restain with nuclear stain.

NOTE: If spheroids are too buoyant for direct imaging, incubate the microplate overnight and then centrifuge 1 to 2 minutes at 200 x g to settle spheroids. It may also be necessary to break the surface tension in the wells by carefully aspirating solution from the wells with a wide-bore pipet tip (Corning TF-205-WB-R-S) and dropping solution back into the well.

Conclusions

- Tissue clearing with Corning 3D Clear reagent follows a simple protocol and can easily be optimized for numerous cell types and microplate formats.
- Corning 3D Clear reagent is compatible with spheroid microplates, allowing culture, processing, and imaging in a single assay plate.
- Tissue clearing with Corning 3D Clear enables visualization of the entire 3D spheroid structure.

References

1. 3D Imaging of Optically Cleared Spheroids in Corning Spheroid Microplates (Corning Lit. Code CLS-AN-509).
2. Corning Spheroid Microplates User Guide (Corning Lit. Code CLS-AN-235).
3. Corning Spheroid Microplates Spheroid Formation Protocol (Corning Lit. Code CLS-AN-308).
4. Considerations for Three-Dimensional Cell Culture when using the Corning Spheroid Microplate (Corning Lit. Code CLS-AN-446).
5. Co-culturing and Assaying Spheroids in the Corning Spheroid Microplate (Corning Lit. Code CLS-AN-390).
6. Citations on Corning Spheroid Microplates with Ultra-Low Attachment Surface (Corning Lit. Code CLS-AN-507).
7. Choi HS, Won T, Hou X, Chen G, Bracamonte-Baran W, Takor MV, et al. Innate lymphoid cells play a pathogenic role in pericarditis. Cell Rep 2020;30(9):2989-3003.e6. doi: 10.1016/j.celrep.2020.02.040.
8. Sargent JA, Roberts VHJ, Gaffney JE, Frias AE. Clarification and confocal imaging of the nonhuman primate placental micro-anatomy. Biotechniques 2019;66(2):79-84. doi: 10.2144/btn-2018-0110.
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