

Cancer Therapeutic Screening in Tumor Spheroids Co-cultured with Fibroblast and Immune Cells Using Corning® Spheroid Microplates

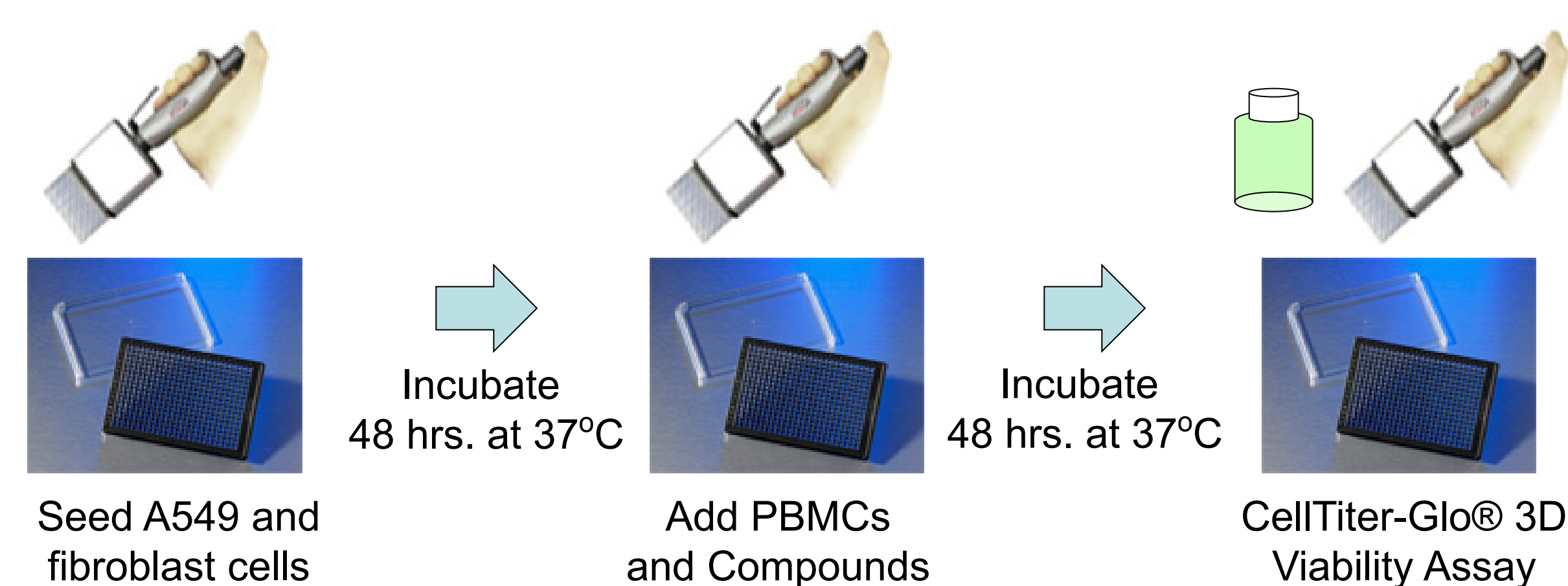
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Abstract

Two-dimensional (2D) cell culture models are frequently used in drug discovery for the development of cancer therapeutics due to their ease of use and established compatibility with high throughput screening. More elaborate, three-dimensional (3D) cell culture models have been developed, which more closely mimic the *in vivo* tumor microenvironment, to bridge the gap between successful *in vitro* studies and success in clinical trials. Understanding the complex interactions between cancer cells and other cell types in the tumor microenvironment, such as fibroblasts, endothelial cells, and immune cells, is critical to predict therapeutic efficacy, as many factors, such as hypoxia, extracellular matrix and gradients of various soluble factors have been associated with both drug resistance and sensitization. However, conventional methods for 3D cell culture are often time consuming, display increased variability, and lack throughput. Corning® spheroid microplates are multiple well, cell culture plates with opaque walls and unique clear, round well-bottom geometry that utilize Corning Ultra-Low Attachment surface coating. The coating is hydrophilic, biologically inert, and non-degradable, which enables the rapid and highly reproducible formation of a single multicellular tumor spheroid, centered in each well. In this study, a tumor cell line was cultured in the Corning spheroid microplates with and without co-culture conditions, including primary fibroblasts and peripheral blood mononuclear cells. Tumor spheroid viability was screened after treatment with various chemotherapeutics in both single tumor cell line culture and co-culture conditions. Dose-dependent responses of selected chemotherapeutics were compared, demonstrating the importance of including multiple cell types in 3D assays to more closely mimic the *in vivo* tumor microenvironment.

Assay Overview



Methods

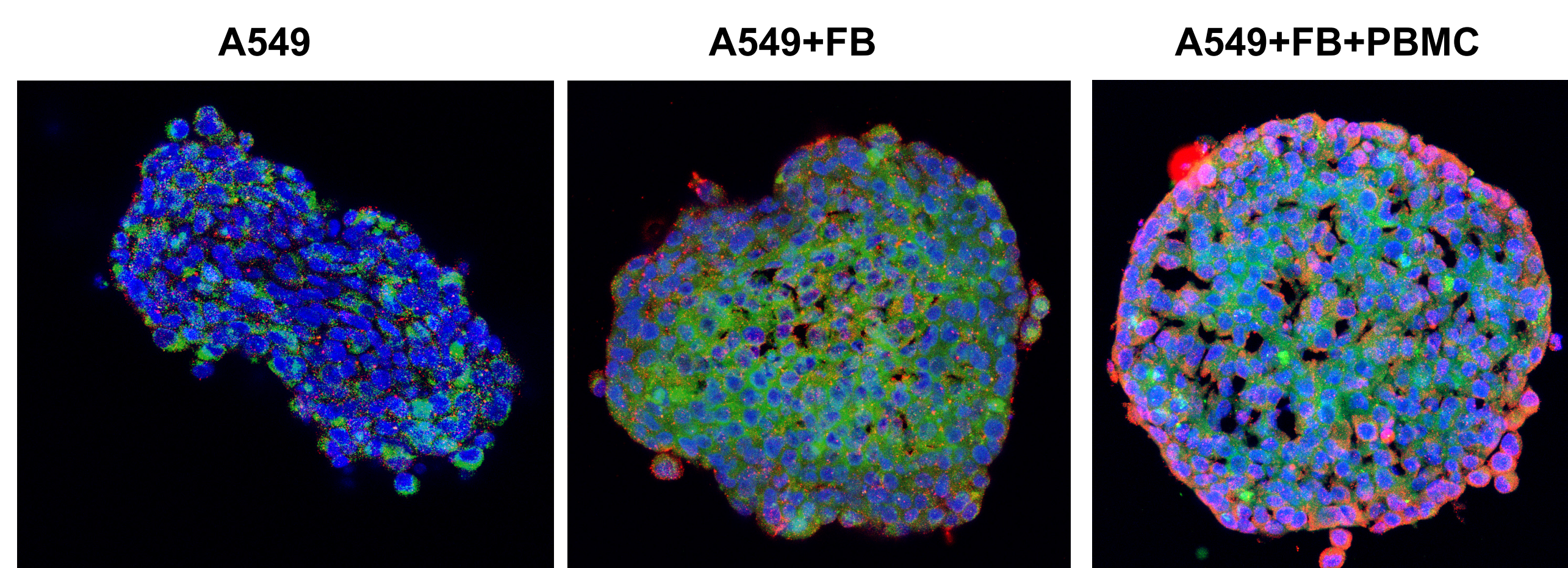
Spheroid Formation and Cell Viability Assays

- A549 (ATCC Cat. No. CCL-185™) and normal human lung fibroblasts [FB] (Lonza Cat. No. CC-2512) were cultured in 2D format following vendors' protocols.
- Confluent cells were harvested and seeded in Corning 384-well spheroid microplates (Corning Cat. No. 3830) at varying seeding densities in mono- and co-culture in 20 μ L fibroblast media (Lonza Cat. No. CC-3132) per well. Spheroid microplates were covered with a breathable membrane sealing tape (Corning Cat. No. 3345) and pulse centrifuged at 130 x g. Spheroids were cultured in a humidified 37°C, 5% CO₂ incubator for 48 hours.
- For tri-culture, 10 μ L of peripheral blood mononuclear cells [PBMC] (All Cells Cat. No. PB003F) in fibroblast media were added per well. For mono- and co-culture conditions, 10 μ L of fibroblast media were added per well.
- Immediately following media or PBMC addition, 10 μ L per well of compounds in 10% dimethyl sulfoxide [DMSO] (Corning Cat. No. 25-950-CQC) were added and mixed twice using a CyBi®-Well pipettor. 10 μ L of 10% DMSO was used as vehicle control.
- Spheroids were cultured for an additional 48 hours prior to imaging and cell viability assays.
- For cell viability assays, cells were assayed in the spheroid microplate using CellTiter-Glo® 3D (Promega Cat. No. G9683) following vendor's protocols. Plates were read on a Tecan Infinite® M1000 plate reader.

Staining/Imaging

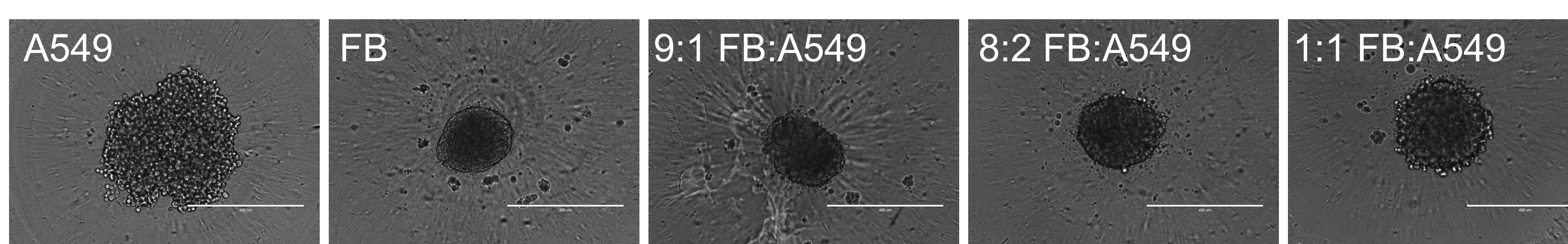
- Media was aspirated from the mono-, co-, and tri-culture spheroids and the spheroids were fixed in 4% paraformaldehyde (Electron Microscopy Sciences Cat. No 157-SP).
- For immunohistochemistry [IHC] staining, fixed cells were embedded and sectioned at 10 μ m via cryostat prior to staining and imaging with a Leica confocal microscope. Cells were stained for anti-cytokeratin 7 (CK7, Abcam Cat. No. ab9021) and anti-fibroblast activation protein (FAP, Abcam Cat. No. ab53066) following vendor's protocols. Immunohistochemistry was performed at the University of New England (Biddeford, Maine)
- For Live/Dead staining, A549 and FB cells were seeded in mono-culture and in co-culture conditions with a fibroblast to A549 ratio of 9:1 with a total of 2K cells per well. After 96 hours of culture, cells were stained using LIVE/DEAD® Viability/Cytotoxicity Kit following vendor's protocols (ThermoFisher Scientific Cat. No. L3224).

Co-Culture Spheroid Formation

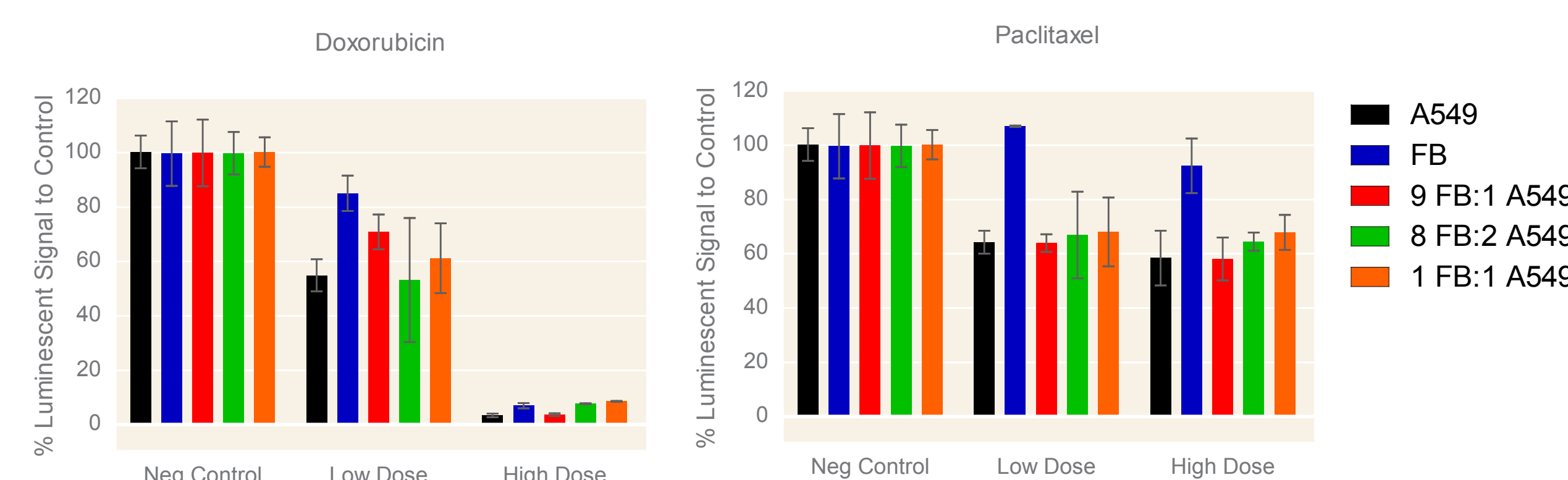


IHC images of mono-, co-, and tri-culture spheroids. A549 cells were seeded at 750 cells/well, A549, and fibroblast cells were seeded at 2K cells/well at a ratio of 1:1, and A549, fibroblast, and PBMCs were seeded at 2K cells/well at a ratio of 2:1:1. Cells were cultured in the spheroid microplate for 96 hours prior to fixation in 4% PFA. Spheroids were stained with DAPI nuclei counterstain (blue), anti-Cy7 (green), and anti-FAP (red).

Co-culture Titration

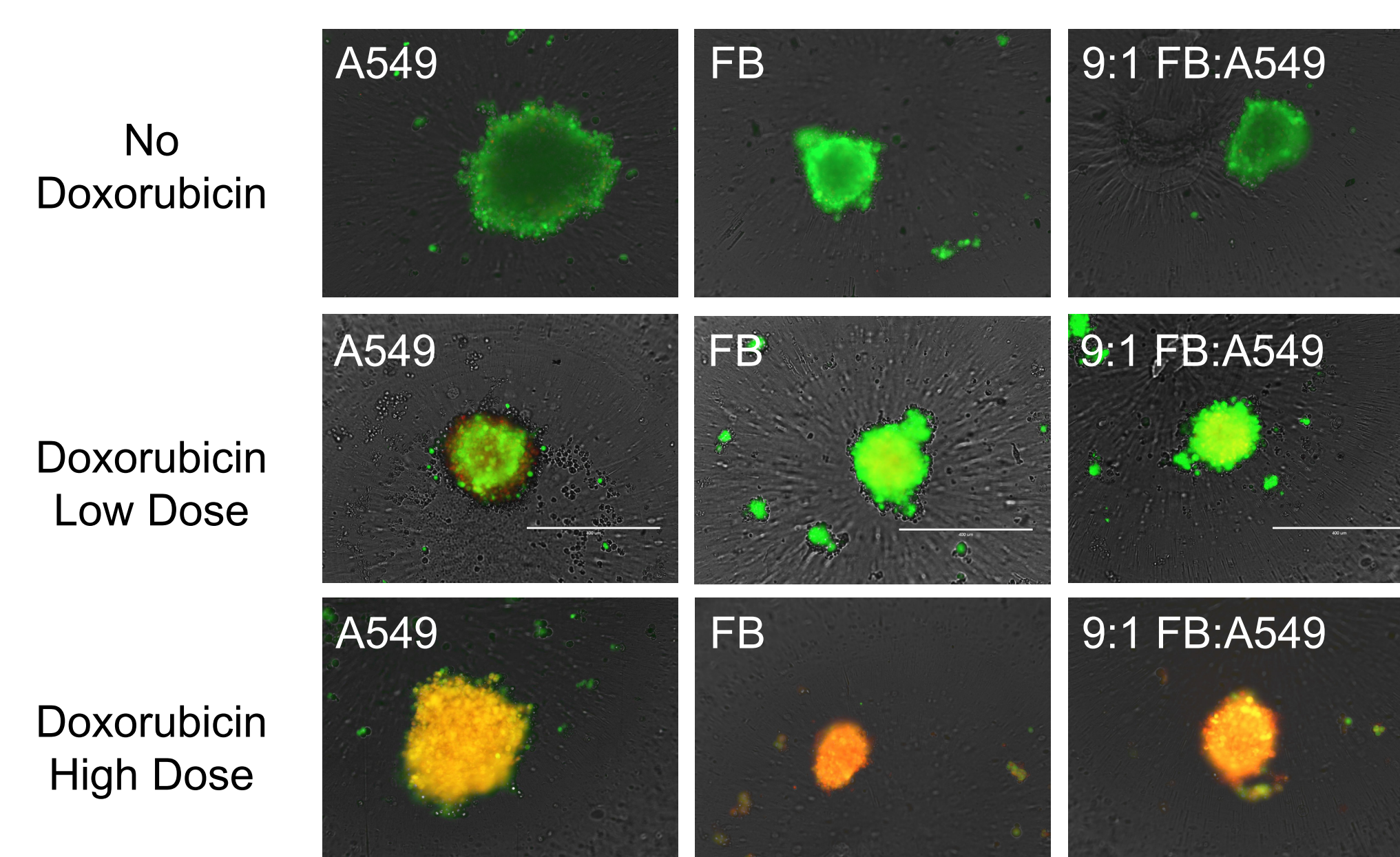


96-hour 3D cultures of mono- and co-culture A549 and lung fibroblast [FB] cells in Corning 384-well spheroid microplates. Cells were seeded in mono-culture and in co-culture ratios of fibroblast to A549 cells of 9:1, 8:2, and 1:1 in a total of 2K cells/well.



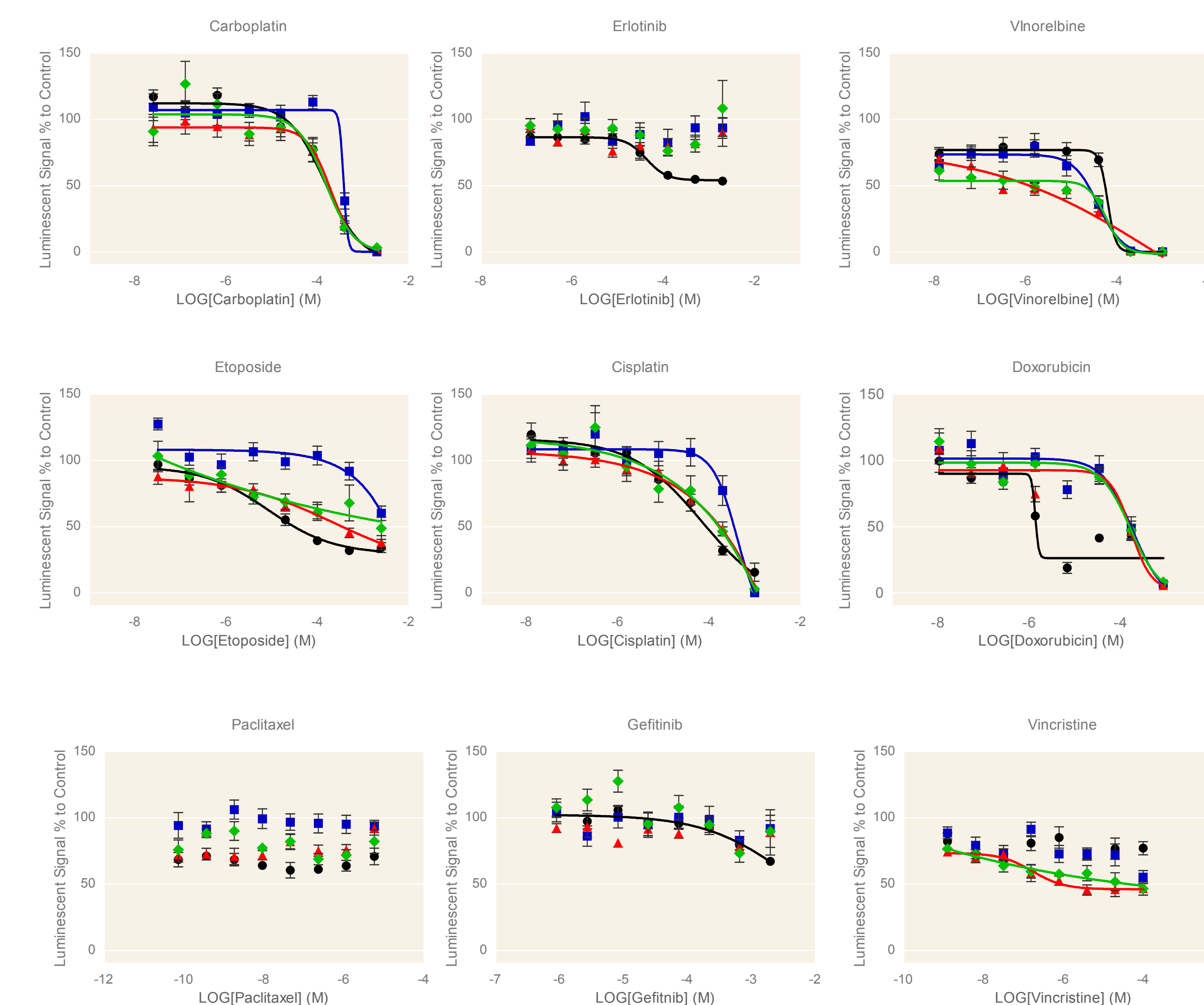
48-hour 3D cultures of mono- and co-culture A549 and lung fibroblast (FB) cells were exposed to doxorubicin (high dose = 862 μ M, low dose = 1.3 μ M) and paclitaxel (high dose = 1.2 μ M, low dose = 9 nM) for 48 hours. FB mono-culture demonstrated higher cell viability after 48-hour exposure to low dose doxorubicin, as measured using CellTiter-Glo 3D cell viability assay, than A549 mono-culture spheroids. A 9:1 ratio of FB to A549 cells also displayed this protective effect from the low dose doxorubicin exposure. FB mono-culture displayed resistance to paclitaxel exposure at both high and low doses compared to the effects seen on A549 mono-culture spheroids. Error bars represent SD.

Live/Dead Staining of Spheroids



Live (green) and dead (red) stained 96 hour 3D cultures of mono- and co-culture A549 and FB cells exposed to high (34.5 μ M) and low (27.6 μ M) doses of doxorubicin or no doxorubicin (vehicle control) for 48 hours in Corning 384-well spheroid microplates. FB mono-culture displayed the most intense live staining upon low dose exposure to doxorubicin, while A549 mono-culture showed increased cell death. The 9:1 ratio of FB to A549 cells also displayed a protective effect at the low dose doxorubicin exposure. All cell types displayed significant toxicity after high dose exposure.

Mono-, Co-, and Tri-culture Response to Chemotherapeutic Compounds



Dose response curves of chemotherapeutic compounds applied to mono-, co-, and tri-culture spheroids for 48 hours were generated using CellTiter-Glo 3D cell viability assay. The presence of fibroblast and immune cells affected the potency of several compounds. Assays were performed in triplicate two independent times. Error bars represent SEM.

■ A549
■ FB
■ A549+FB
■ A549+FB+PBMC

Compound	A549 TC ₅₀ (M)	FB TC ₅₀ (M)	A549+FB TC ₅₀ (M)	A549+FB+PBMC TC ₅₀ (M)
Carboplatin	1.48E-4	3.75E-4	1.95E-4	1.53E-4
Erlotinib	4.25E-5	N/A	N/A	N/A
Vinorelbine	6.26E-5	3.87E-5	6.92E-4	5.72E-5
Doxorubicin	1.38E-6	1.83E-4	1.70E-4	1.69E-4
Etoposide	8.51E-6	>5.00E-4	>5.00E-4	>5.00E-4
Cisplatin	7.07E-5	>2.00E-4	>2.00E-4	>2.00E-4
Paclitaxel	N/A	N/A	N/A	N/A
Gefitinib	>2.00E-3	N/A	N/A	N/A
Vincristine	N/A	N/A	N/A	N/A

Toxicity potency (TC₅₀) values of chemotherapeutic compounds applied to mono-, co-, and tri-culture spheroids for 48 hours were generated using CellTiter-Glo 3D cell viability assay. Although the presence of fibroblast and immune cells did not affect the potency of compounds carboplatin and cisplatin to A549 lung tumor cells, a right-shift in the potency of several other known chemotherapeutics was observed.

Conclusions

- The Corning 384-well spheroid microplate can be used to generate and perform viability assays with co-culture spheroids in an easy-to-use, high throughput format.
- Spheroids formed and cultured in the Corning spheroid microplate are amenable to immunohistochemical and fluorescent staining analysis.
- The presence of other cell types with tumor cells in a spheroid affects the potency of several chemotherapeutic compounds.