Clonogenic Survival Analysis of 3D Bioprinted Irradiated Tumor Constructs

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ABSTRACT

Purpose
- Develop and validate a workflow for clonogenic survival assay in extrusion-based 3D bioprinted cancer constructs following irradiation.

Methods
- A549 cells were encapsulated in alginate/gelatin hydrogel, bioprinted in an “S” shape.
- They were cultured 14 days, irradiated (0, 2, 4, 8 Gy), decrosslinked, seeded for clonogenic assay.
- Colonies stained (crystal violet), quantified via an in-house semi-automated custom web-based colony-counting tool.

Results
- The control group plating efficiency was 14.5% ± 0.6% (n = 3 biological replicates)
- Surviving fraction (SF) was found to decrease in a linear-quadratic fashion
- SF at 2 Gy is in the lower quartile of 2D A549 studies
- Dose at which SF = 10% (D10) is less than 2D studies
- One bioprint was a statistical outlier prior to pooling (see Discussion)

Conclusions
- A reproducible clonogenic workflow was established for 3D bioprinted constructs
- Our A549 3D model yielded SF values that are in the lower quartile of 2D in vitro models

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INTRODUCTION

- Radiation therapy is used in ~50% of cancer patients; accurate in vitro radiosensitivity data are essential for research and treatment optimization[1]
- The clonogenic survival assay is the gold standard for radiation-induced reproductive cell death but is traditionally performed in 2D monolayer culture[2]
- 2D models lack the 3D architecture, extracellular matrix interactions, oxygen gradients, and cell-cell signaling of real tumors, limiting translational relevance[3]
- 3D bioprinted constructs enable spatially controlled, reproducible tumor-mimicking architectures at clinically relevant cell densities4
- To our knowledge, no protocol previously existed for clonogenic assays on cells recovered from irradiated 3D bioprinted constructs

Objective:
- Develop an end-to-end workflow bridging 3D bioprinting with clonogenic survival analysis following irradiation.

METHODS AND MATERIALS

Cell Culture & Bioprinting
- A549 human lung adenocarcinoma cells; passages 15–17
- Three biological replicates were constructed
- Bioink: 2% alginate + 3% gelatin, ~3×10^6 cells/mL
- Extrusion bioprinting, 25-gauge needle; “S” shape, 5 × 5 × 0.3 mm (Fig. 1)
- Crosslinked (100 mM $CaCl_2$), matured 14 days before irradiation

Irradiation
- 0, 2, 4, 8 Gy at ~1 Gy/min via CellRad irradiator (Precision X-ray, Inc.); 100 kV and 2mm Al added filtration

Recovery & Clonogenic Assay
- Decrosslinked (50 mM sodium citrate), cell count by trypan blue using Celldrop (DeNovix, Inc.)
- Dose-adjusted seeding: 600 (0 Gy), 800 (2 Gy), 2,000 (4 Gy), 12,000 (8 Gy) cells/well
- 10–14 day incubation; fixed methanol, stained 0.5% crystal violet

Quantification
- In-house semi-automated web-based colony-detection tool, with manual review to select/deselect colonies and remove artifacts
- Colony defined as ≥ 50 cells.
- Plating Efficiency = colonies/cells seeded × 100% at 0 Gy; SF = (colonies/cells seeded)/PE
- Nested design: n = 3 biological replicates (by cell passage 15/16/17), each containing 1–3 technical replicates (bioprints)

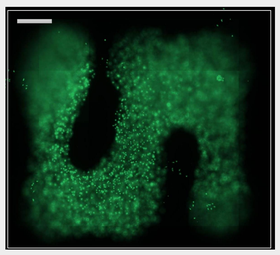

Figure 1. Live-cell fluorescence image of the 3D bioprinted "S"-shaped A549 construct (Pico, Molecular Devises, Inc.), showing viable cell distribution (green, calcein-AM) throughout the hydrogel. Scale bar = 952.88 µm.

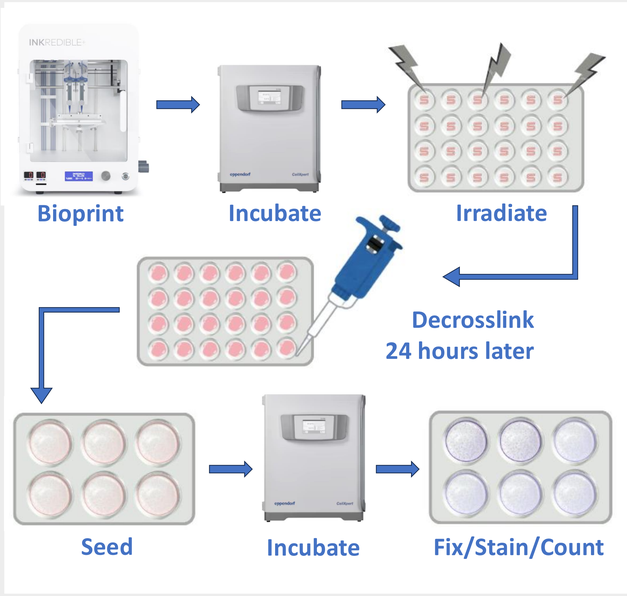

Figure 2. Clonogenic assay workflow for 3D bioprinted A549 constructs.

RESULTS

- n = 3 biological replicates assayed at 0, 2, 4, 8 Gy.
- Plating efficiency at 0 Gy ranged 8.5–15.1% across bioprints — Print 1 was a clear outlier at 8.5% (see Discussion, Fig. 3).

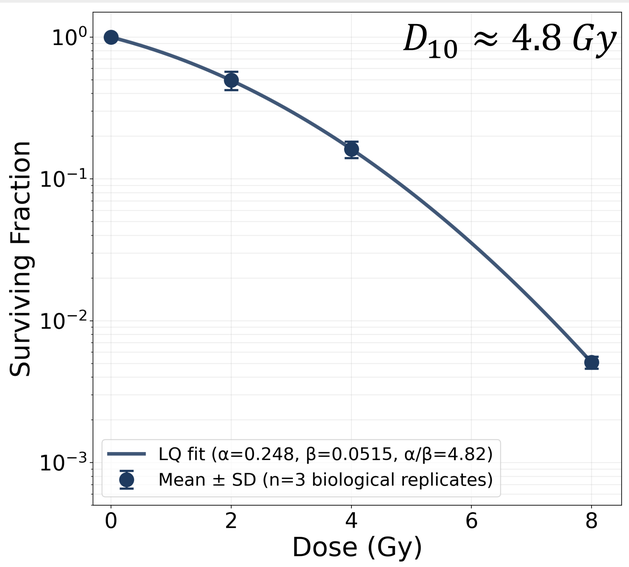

Figure 3. Survival curve, mean ± standard deviation (SD) for n = 3 biological replicates; technical replicates averaged within each biological replicate before pooling). LQ fit via log-linear regression.

Dose (Gy)	SF
0	1.00
2	0.49 ± 0.07
4	0.16 ± 0.02
8	0.0051 ± 0.0005

Table 1. Surviving fraction ± SD by dose (n = 3 biological replicates; technical replicates nested within each).

α	β	α/β
0.248 ± 0.002	0.0515 ± 0.0003	4.82 ± 0.07

Table 2. Linear-quadratic fit parameters (α, β, α/β) with standard error, from log-linear regression on the pooled survival data (n = 3 biological replicates).

DISCUSSION

Print-to-Print Variability
- Baseline plating efficiency varied across bioprints (13.2–15.1%), despite identical protocol and same-day printing
- Points to a hydrogel batch or print-order effect — not random biological noise, since wells within a print agreed closely

Print 1 Outlier
- PE was roughly half of the other five bioprints; distinct dose-response shape
- Cell passage was ruled out as the explanation (shared passage 15 with two typical prints)
- Excluded from the pooled analysis as a documented, flagged outlier

3D vs. 2D Radiosensitivity
- 3D bioprinted constructs showed overall SF in the lower quartile of 2D A549 in vitro studies,[5] likely reflecting bioprinting/recovery stress

Limitations & implications
- Only 3 non-zero dose points per bioprint limited LQ curve resolution at the individual-print level
- Single cell line limits generalizability

CONCLUSIONS

- A reproducible, end-to-end workflow was established for clonogenic survival assessment in 3D bioprinted tumor constructs after irradiation
- A549 constructs showed a clear linear-quadratic dose-dependent survival (Fig. 3 & Table 1)

Future Directions
- Increase bioprint replicates and add intermediate dose points (1, 6 Gy)
- Post-print viability and hypoxia assays to isolate bioprinting stress versus presence of hypoxia
- Identify the source of Print 1-type outlier events (hydrogel batch, print order)
- Hypoxic and co-culture 3D construct models.
- Patient-derived tumor cell lines

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