

Assessment of Doxorubicin Internalization and Cytotoxicity in Primary Tumor Spheroids After Collagen Digestion

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The limited penetration of chemotherapeutic agents into solid tumors remains a major obstacle to effective cancer treatment and is strongly influenced by the extracellular matrix (ECM). Three-dimensional (3D) tumor spheroids derived from primary tumor cells (PTCs) represent a valuable in vitro model to study how ECM composition and organization regulate drug distribution and cytotoxicity with accurate physiological relevance. By recapitulating key features of the tumor microenvironment, including endogenous collagen deposition and diffusion-limited drug accessibility, these models enable mechanistic investigation of microenvironment-driven drug resistance. Here, we describe a set of integrated protocols to evaluate the impact of ECM remodeling on chemotherapeutic response in primary breast tumor spheroids. Spheroids are generated from isolated primary tumor cells and subjected to controlled enzymatic degradation of collagen-rich ECM using ultrapure recombinant collagenases. As a model chemotherapeutic agent, doxorubicin is employed thanks to its widespread clinical use, well-characterized cytotoxic mechanism, and intrinsic fluorescence, which allows direct visualization of drug uptake and spatial distribution by confocal microscopy. In parallel, drug-induced cytotoxicity is quantified using a luminescent 3D viability assay. Together, these protocols provide a reproducible and accessible platform for investigating ECM-mediated barriers to drug delivery and toxicity in 3D tumor models. This framework facilitates the implementation of physiologically relevant assays to study drug efficacy and therapeutic resistance in solid tumors. © 2026 The Author(s). *Current Protocols* published by Wiley Periodicals LLC.

Basic Protocol 1: Induction of breast cancer, isolation of tumor primary cells, and generation 3D spheroids

Support Protocol: Culture and storage of PTCs

Basic Protocol 2: Evaluation of collagen expression and enzymatic degradation in primary tumor spheroids by confocal microscopy

Basic Protocol 3: Enzymatic degradation of the endogenous matrix collagen in primary tumor spheroids using recombinant collagenases

Basic Protocol 4: Assessment of doxorubicin uptake in spheroids by confocal microscopy

Basic Protocol 5: Assessment of cell viability in doxorubicin-treated spheroids using CellTiter-Glo 3D assay

Lo Cicero et al.

1 of 19

Keywords: collagenase • cytotoxicity • doxorubicin • extracellular matrix • tumor spheroids

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INTRODUCTION

The efficacy and accessibility of anticancer agents are deeply influenced by the tumor microenvironment, particularly by the composition and density of the extracellular matrix (ECM) (Desai et al., 2025; Lo Buglio et al., 2024). The ECM acts as both a physical barrier and a biochemical modulator of drug diffusion, reducing intratumoral drug penetration and contributing to chemoresistance (Yuan et al., 2023). Understanding how ECM remodeling affects drug penetration and cytotoxicity is, therefore, a central challenge in preclinical toxicology and drug development in solid tumors (Huang et al., 2021a).

In this context, preclinical strategies have been proposed to modulate or degrade the ECM, including the *in vivo* use of proteolytic enzymes such as collagenases, either in free form or delivered via drug delivery systems (García-Olmo et al., 2021; J. Li et al., 2023; J. Wang et al., 2021; Y. Wang et al., 2024; Zhao et al., 2025).

Traditional two-dimensional (2D) monolayer cultures are limited in their ability to mimic these processes *in vitro*, as they lack the architectural complexity and molecular gradients found in solid tumors. In contrast, three-dimensional (3D) tumor spheroids more closely recapitulate key features of *in vivo* tumors, including oxygen and nutrient gradients, intercellular communications, and diffusion-limited drug accessibility (Arora et al., 2024; Campora & Lo Cicero, 2025; Rodrigues et al., 2024). However, tumor spheroids composed exclusively of cancer cells fail to accurately reproduce the complexity and heterogeneity of the tumor microenvironment, particularly the structural and biochemical features of the ECM. To overcome these limitations, advanced 3D models that incorporate stromal components, such as fibroblasts and endothelial cells (e.g., HUVECs) or microvascular fragments (Cortini et al., 2023; A. Li et al., 2022; Lo Cicero et al., 2026; Parashar et al., 2022) have been developed, allowing a more accurate representation of tumor–stroma interactions. Notably, the use of primary cells isolated directly from tumor tissues has enabled the formation of models capable of producing an endogenous ECM enriched in type I collagen and expressing a full repertoire of proteins involved in matrix deposition, degradation, and cellular remodeling (Lo Cicero et al., 2025). These complex multicellular systems are crucial for a more comprehensive understanding of tumor biology and provide highly relevant platforms for studying drug penetration, accumulation, and toxicity in a more physiologically relevant context (Breslin & O'Driscoll, 2016; Lo Cicero et al., 2025; Stock et al., 2016).

The protocols presented here describe an integrated workflow for evaluating ECM remodeling and chemotherapeutic response in primary tumor spheroids. Spheroids are first generated from freshly isolated tumor cells, which produce their own ECM. To investigate how the ECM modulates drug uptake and cytotoxicity, spheroids are treated with ultrapure recombinant collagenases (Salamone et al., 2014; Volpe et al., 2016) to degrade collagen networks and enzymatically alter matrix density. Following this controlled ECM

disruption, the anthracycline doxorubicin is used as a chemotherapeutic model, thanks to its intrinsic fluorescent properties, which enable direct assessment of drug penetration, retention, and cytotoxic effects.

Drug uptake is monitored over time by confocal microscopy through z-stack image acquisitions of the spheroid. In parallel, cell viability is assessed using the CellTiter-Glo 3D luminescent assay, as an indicator of metabolic activity and drug-induced cytotoxicity.

Together, these methods provide a reproducible and physiologically relevant platform for investigating how ECM degradation alters drug accessibility and toxicological outcomes in 3D tumor systems. This approach enables the modeling of ECM-mediated drug distribution barriers, quantitative assessment of treatment efficacy, and exploration of how resistance mechanisms change through matrix remodeling. Therefore, the combination of collagenase treatment and doxorubicin exposure represents a versatile system for studying both drug delivery and toxicity in a realistic tumor-like environment.

STRATEGIC PLANNING

The following protocols (Fig. 1) are designed to isolate rat tumor primary cells and develop a 3D model to investigate how ECM degradation by ultrapure recombinant collagenase influences the toxicological response to doxorubicin in a 3D tumor microenvironment. Collagenase concentration and incubation time must be carefully optimized to achieve partial matrix digestion without disrupting spheroid integrity. In spheroids treated with collagenase, doxorubicin can be used in a lower concentration to achieve cytotoxicity levels, thereby reducing the drug-related off-target toxicity. The combination of confocal imaging and luminescent viability assays provides both spatial and quantitative data, offering a comprehensive view of drug penetration, accumulation, and efficacy within 3D cultures. Together, these methodologies provide a powerful experimental framework for bridging the gap between in vitro cellular toxicology and in vivo pharmacodynamics outcomes.

NOTE: All animal procedures must be reviewed and approved by the institutional Animal Care and Use Committee and must comply with national regulations governing the care and use of laboratory animals.

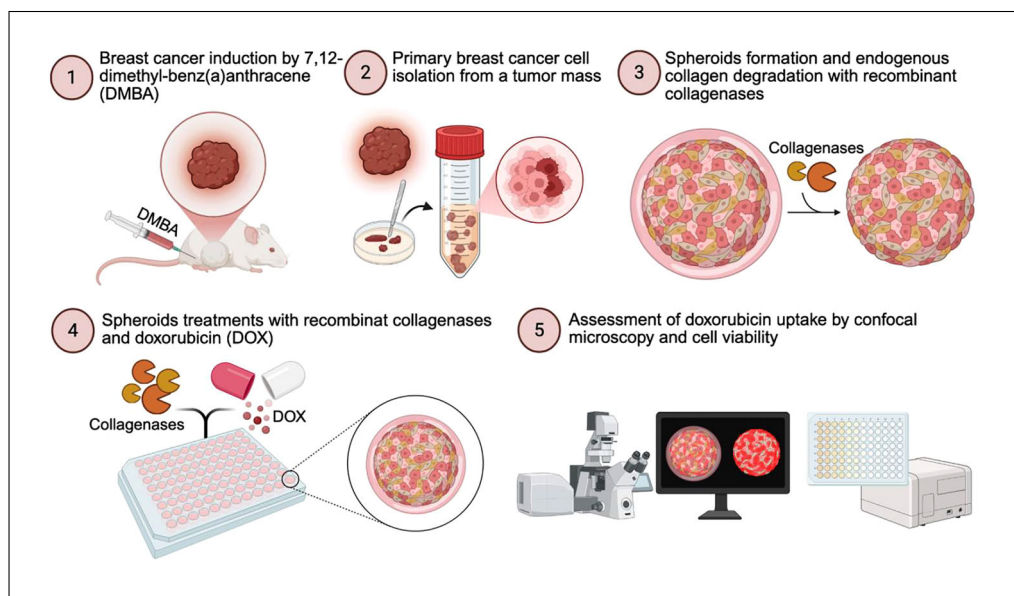


Figure 1 Schematic workflow of Basic Protocols 1 to 5.

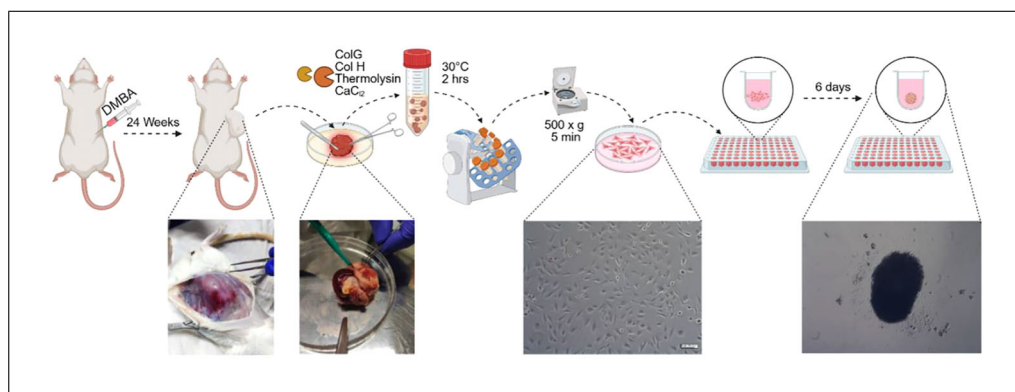


Figure 2 Schematic representation describing the induction of breast cancer with DMBA and tissue digestion to isolate cells. Tumor mass is excised and treated with recombinant collagenases (COLG and COLH), thermolysin, and CaCl_2 . Isolated cells are seeded in low attachment conditions to generate spheroids.

NOTE: All isolation, culture, and treatment steps must be performed under sterile conditions, preferably within a class II biosafety cabinet.

BASIC PROTOCOL 1

INDUCTION OF BREAST CANCER, ISOLATION OF TUMOR PRIMARY CELLS, AND GENERATION OF THREE-DIMENSIONAL SPHEROIDS

This protocol describes the isolation of primary cells from a tumor mass (PCTM) induced in an immunocompetent female Wistar rat and the subsequent generation of three-dimensional (3D) spheroids. The breast tumor mass was induced by treatment with 7,12-dimethylbenz[a]anthracene (DMBA). After six months, the tumor mass was excised and enzymatically digested using recombinant collagenases and thermolysin to isolate primary cells, which were then used to generate 3D spheroids (Fig. 2).

Materials

55-day-old female Wistar rats ($n = 1$, ENVIGO RMS)

Authorization number 523/2022-PR, issued according to art. 31 del D.lgs. 26/2014 Istituto Superiore di Sanità, Italy.

Rat food

Isoflurane (Zoetis Italia, cat. no. 103287025)

7,12-dimethyl-benz(a)anthracene (DMBA) (Sigma-Aldrich, cat. no. D3254)

Corn oil (Sigma-Aldrich, cat. no. C8267)

70% (v/v) ethanol in dH_2O (Carlo Erba, cat. no. 414632)

Dulbecco's modified Eagle's medium, high glucose (HG-DMEM) (Sigma-Aldrich, cat. no. D6546)

Penicillin streptomycin (Euroclone Celbar, cat. no. ECB3001)

Digestion buffer (see recipe)

Collagen type I (Sigma-Aldrich, cat. no. CLS354236) in 0.02 N acetic acid

Fetal bovine serum (FBS) (Euroclone, cat. no. ECB3052D)

DMEM/F12 (Sigma-Aldrich, cat. no. D6421)

L-glutamine (Euroclone Celbar, cat. no. ECB3000D)

Cage equipped with an efficient particulate air (EPA) filter

Equipment for rat anesthesia and euthanasia

1-ml syringe with 27-G needle

Calipers

Surgical instruments (blunt-tip fine tweezers, small fine-point scissors, and scalpel), sterile

15- and 50-ml conical tubes, sterile

Ice container and ice
 Class II biosafety cabinet
 100-mm Petri dishes, sterile
 10- and 25-ml pipets, sterile
 Rotating mixer
 Tissue culture microscope
 37°C incubator and/or water bath
 Standard tabletop centrifuge
 Ultra-low attachment, U-bottom 96-well plates, sterile (Greiner, cat. no. 650970)
 Filtered pipette tips, sterile

1. House a 7-week-old female Wistar rat (body weight around 150 to 200 g) in a cage equipped with an EPA filter. Provide food ad libitum and maintain the temperature at $23^{\circ} \pm 0.5^{\circ}\text{C}$ and relative humidity at $50\% \pm 5\%$. Set a 12 hr light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m.). Allow 7 days of quarantine followed by 7 days of acclimatization.
2. Anesthetize the rat by inhalation of 2% isoflurane.
3. Prepare 7,12-dimethyl-benz(a)anthracene (DMBA) by resuspending it in corn oil at a concentration of 20 mg/ml. Using a 1-ml syringe, inject the solution intraperitoneally at a dose of 50 mg/kg.

For example, for a 7-week-old female Wistar rat weighing 170 g, administer 425 μl of the DMBA solution into the left fourth mammary gland.

4. Monitor tumor growth weekly using calipers until the tumor reaches $\sim 80 \text{ mm}^3$ (around week 24).
5. Anesthetize the rat again with 2% isoflurane and euthanize it by cervical dislocation.
6. Dissect the tumor mass using sterile surgical instruments (blunt-tip fine tweezers, small fine-point scissors, and a scalpel). Immediately place the tissue into a 50-ml conical tube containing 70% ethanol for 2 to 3 min. Transfer the tissue to a new 50-ml conical tube containing HG-DMEM supplemented with 300 U/ml penicillin and 300 U/ml streptomycin and keep it on ice.
7. Transfer the sample to a sterile 100-mm Petri dish under aseptic conditions in a class II biosafety cabinet. Clean and mince the tissue using sterile blunt-tip fine tweezers, small fine-point scissors, and a scalpel.
8. Transfer small tissue fragments ($\sim 1 \text{ g}$ total) into a 15-ml conical tube containing 10 ml of prewarmed (37°C) digestion buffer. Using a sterile 25-ml pipette, dissociate the tissue by gently pipetting up and down. Incubate the sample at 30°C for 2 hr in a rotating mixer.

Depending on tissue fragment size, digestion may be facilitated by intermittently pipetting the suspension up and down with a sterile 25-ml pipette. Perform this step in the class II biosafety cabinet.

Depending on the tissue fragment size and the density of the tumor mass, reduce the incubation time if the tissue appears digested by analyzing it on the optical microscope.

9. Prepare collagen-coated Petri dishes (four dishes per 1 g tissue sample). Dilute the collagen solution in 0.02 N acetic acid to a final concentration of 50 $\mu\text{g}/\text{ml}$ under the class II biosafety cabinet. Add 5 ml of the collagen solution to each Petri dish and incubate at 37°C for 2 hr. Remove the collagen solution and wash each dish twice with 5 ml HG-DMEM.

10. From step 8, stop the tissue digestion by adding HG-DMEM containing 300 U/ml penicillin, 300 U/ml streptomycin, and 10% (v/v) FBS.
11. Perform the following:
 - a. Centrifuge the digested samples 5 min at $500 \times g$, room temperature.
 - b. Return to the class II biosafety cabinet and discard the supernatant.
 - c. Resuspend the cell pellet in 10 ml HG-DMEM supplemented with 300 U/ml penicillin, 300 U/ml streptomycin, and 10% (v/v) FBS, then centrifuge again 5 min at $500 \times g$, room temperature.
 - d. Repeat this wash step three times.
 - e. Resuspend the cell pellet in 12 ml DMEM/F12 supplemented with 300 U/ml penicillin, 300 U/ml streptomycin, and 10% (v/v) FBS.
12. Seed 4 ml of the cell suspension onto each collagen-coated Petri dish prepared in step 9 and add 4 ml of DMEM/F12 medium supplemented with 300 U/ml penicillin, 300 U/ml streptomycin, 2 mM L-glutamine, and 10% (v/v) FBS. Examine the cultures using an inverted light microscope.

Small tissue fragments may remain in the suspension. Leave these fragments in the Petri dish, as additional cells may migrate out over time.

13. Incubate the cultures overnight at 37°C in a humidified incubator with 5% CO₂.
14. The following day, remove the medium containing residual tissue fragments, wash the cultures three times with 5 ml DMEM/F12, and incubate again overnight at 37°C with 5% CO₂.

The next day, the culture medium typically appears yellow.

15. Maintain primary cells derived from a tumor mass (PCTM) in HG-DMEM supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 10% (v/v) FBS. Incubate cultures at 37°C in a humidified 5% CO₂ atmosphere for at least 15 passages.
16. To generate spheroids, seed PCTM cells at 1×10^4 cells per well in ultra-low attachment, U-bottom 96-well plates. Incubate plates at 37°C in a humidified 5% CO₂ atmosphere for spheroid formation (24 hr).

If ultra-low attachment plates are unavailable, use standard U-bottom 96-well plates coated with agarose. To prepare them, make a 1.5% (w/v) agarose solution in HG-DMEM (final volume no more than 5 ml/vial) and sterilize it by autoclaving at 121° to 134°C. Under sterile conditions in a class II biosafety cabinet, maintain the agarose solution at 60° to 70°C in a water bath and promptly dispense 40 µl of the agarose solution into each well of the U-bottom plate by using sterile filtered pipette tips. Allow the agarose to solidify at room temperature for ~15 min before seeding cells.

SUPPORT PROTOCOL

CULTURE AND STORAGE OF PRIMARY TUMOR CELLS

This protocol describes the procedures for culturing, maintaining, and cryopreserving primary tumor cells (PTCs) isolated in Basic Protocol 1, as well as the generation of 3D spheroids under ultra-low attachment conditions. The 3D spheroids were monitored over time by optical microscopy to assess spheroid size and morphology.

Materials

Primary tumor cells (PTCs) isolated in Basic Protocol 1
 Complete HG-DMEM (see recipe)
 Dulbecco's phosphate-buffered saline (DPBS) (Sigma-Aldrich, cat. no. D8537)
 1× trypsin-EDTA solution (Sigma-Aldrich, cat. no. T2601)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. D4540)
FBS (Euroclone, cat. no. ECB3052D)

37°C incubator and/or water bath
Class II biosafety cabinet
15-ml conical tubes, sterile
Standard tabletop centrifuge
T125 culture flask
Cryotubes
Controlled-rate freezing container

Thawing PTCs

1. Thaw a vial of PTCs in a 37°C bead bath until only a small ice crystal remains.
2. Under a class II biosafety cabinet, transfer the cell suspension into a 15-ml conical tube containing 7 ml of complete HG-DMEM.
3. Centrifuge the cells 5 min at $300 \times g$, room temperature, and carefully discard the supernatant.
4. Resuspend the cell pellet in 7 ml complete HG-DMEM and transfer the suspension to a culture T125 culture flask.
5. Incubate cells at 37°C and 5% CO₂.
6. Change medium every 2 to 3 days.

Cryopreservation of PTCs

7. Aspirate the culture medium from the T125 culture flask containing PTCs and wash the cells with 5 ml DPBS without Ca²⁺ and Mg²⁺.
8. Add 1 ml of $1 \times$ trypsin-EDTA solution and incubate at 37°C for 5 min.

Gently tap or rock the culture flask to facilitate cell detachment.

9. Neutralize trypsin activity by adding 5 ml of complete HG-DMEM, then transfer the cell suspension to a sterile 15-ml conical tube.
10. Centrifuge the cells 5 min at $300 \times g$, room temperature, and carefully discard the supernatant.
11. Resuspend the cell pellet in FBS supplemented with 10% (v/v) DMSO at a density of 1×10^6 cells per cryovial and aliquot 1 ml per cryovial.

Keep cells on ice during DMSO addition. The number of cryovials depends on the total cell yield.

12. Place cryovials in a controlled-rate freezing container at -80°C for 24 hr, then transfer them to a liquid nitrogen storage tank for long-term storage.

EVALUATION OF COLLAGEN EXPRESSION IN PRIMARY TUMOR SPHEROIDS WITH CONFOCAL MICROSCOPY

In this protocol, multiple spheroids are fixed within a single tube, preserving their 3D architecture throughout all processing steps. The principal steps, including fixation, blocking, and antibody incubations, are all performed under slow rotation. This gentle agitation facilitates uniform exposure of the spheroid surface to reagents and prevents sedimentation, ensuring even staining throughout the structure. This approach enables comprehensive visualization of entire spheroids by acquiring z-stack images using a confocal microscope (Fig. 3). To ensure effective penetration of reagents into the core of the spheroids,

BASIC PROTOCOL 2

Lo Cicero et al.

7 of 19

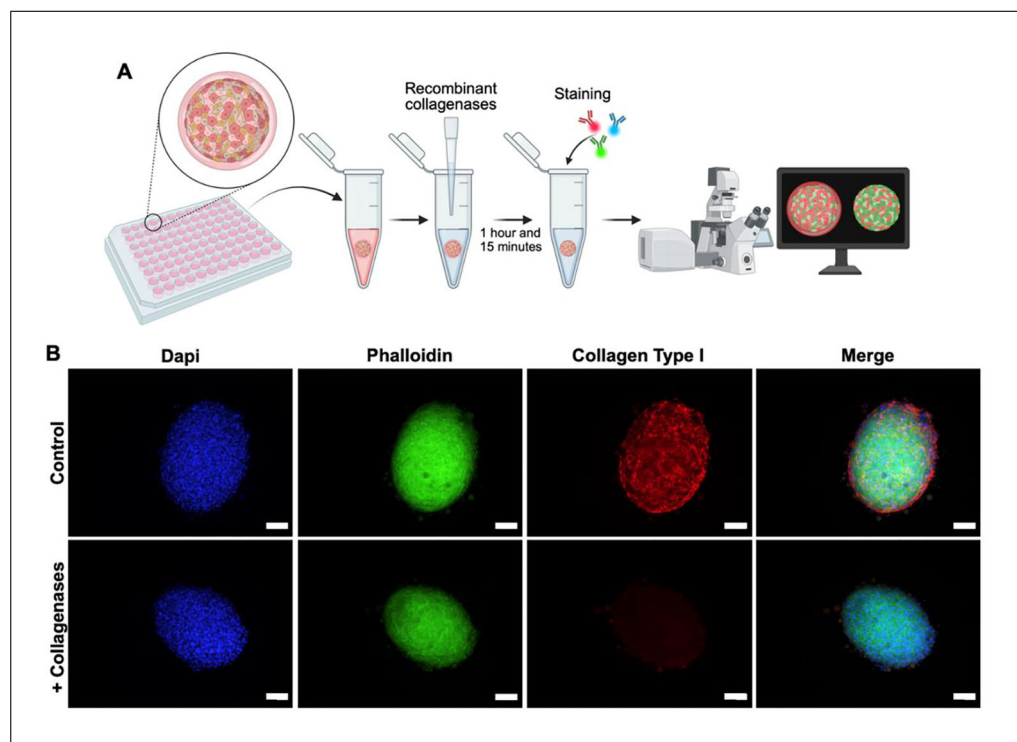


Figure 3 Immunostaining of primary tumor cells spheroids after collagenase treatments. **(A)** Workflow for spheroid treatments with recombinant collagenase and analysis. **(B)** Primary tumor spheroids, untreated (control) and treated with recombinant collagenases COLG and COLH (+ collagenases) for 75 min. Spheroids were labeled with phalloidin-FITC (green), antibody anti-collagen type I (red), and DAPI (blue). Scale bar, 100 μ m.

incubation times with fixatives and antibodies are extended compared to standard immunofluorescence staining protocols.

Materials

Primary tumor cells (PTCs) (from Basic Protocol 1)
 DPBS (Sigma-Aldrich, cat. no. D8537)
 Formaldehyde fixative (see recipe)
 Primary antibody, anti-collagen type I (Millipore, cat. no. 234167)
 Blocking solution (see recipe)
 Fluorescent-labeled secondary antibody, anti-rabbit TRITC (Sigma-Aldrich, cat. no. T6778)
 Phalloidin-FITC (Sigma-Aldrich, cat. no. P5282)
 DAPI (Sigma-Aldrich, cat. no. D9542)
 Mounting medium: 80% (v/v) glycerol (Sigma-Aldrich, cat. no. 15524), 0.01% (w/v) NaN_3 (Sigma-Aldrich, cat. no. S2002) in 0.2 M Trizma base (Sigma-Aldrich, cat. no. T1503) in HCl (Sigma-Aldrich, cat. no. 258148), pH 7.5
 Ultra-low attachment, U-bottom 96-well plates, sterile (Greiner, cat. no. 650970)
 1.5-ml tubes
 Pipettes and sterile filter tips
 Rotator
 Coverslips and glass slides
 Confocal microscope (e.g., Olympus FV10i)
 ImageJ software (NIH)

1. Recover spheroids from the wells of the sterile ultra-low attachment, U-bottom 96-well plates, after 6 days post-seeding in (as described in Basic Protocol 1), and place them in 1.5-ml tubes.

More than one spheroid can be collected in one tube. Use a p1000 pipette to avoid the disruption of the structure.

2. Remove the medium and rinse the spheroid with 500 μ l DPBS.
3. Add 1 ml of 3.7% formaldehyde and fix for 1 hr in slow rotation at room temperature.

Fixation can also be performed with 4% paraformaldehyde.

4. Remove formaldehyde and rinse three times with 500 μ l DPBS.
5. Block with blocking solution for 1 hr in rotation at room temperature.
6. Remove the blocking solution and add 300 μ l of anti-collagen type I antibody diluted 1:20 in the blocking solution. Perform the primary antibody incubation overnight at 4°C in slow rotation.
7. Wash three times in DPBS to remove residual antibody.

Be careful because after the incubation, some spheroids can be placed in the cap of the tube. Gently recover them using a p1000 pipette with DPBS.

8. Incubate with specific fluorescent-labeled secondary antibody, anti-rabbit TRITC, diluted 1:200 in blocking solution containing phalloidin-FITC (diluted 1:500) for 2 hr in rotation at room temperature.
9. Wash three times in DPBS to remove residual antibody and phalloidin.

Be careful because after the incubation, some spheroids can be placed in the cap of the tube. Gently recover them using a p1000 pipette with DPBS.

10. Incubate with DAPI diluted in DPBS (1:20,000) for 10 min in rotation at room temperature.

Be careful because after the incubation, some spheroids can be placed in the cap of the tube. Gently recover them using a p1000 pipette with DPBS.

11. Wash three times in DPBS to remove residual DAPI.
12. Remove any excess DPBS, leaving only 50 μ l.
13. In a slide, place 10 μ l of mounting medium and 10 μ l DPBS containing spheroids (using a p1000 pipette). Place a coverslip and remove the excess liquid with paper.
14. Analyze the immunofluorescence using a confocal microscope and ImageJ software.

ENZYMATIC DEGRADATION OF THE ENDOGENOUS COLLAGEN IN PRIMARY TUMOR SPHEROIDS USING RECOMBINANT COLLAGENASES

This protocol describes the enzymatic degradation of the endogenous extracellular matrix in primary breast tumor spheroids using recombinant collagenases class I (COLG) and class II (COLH) (Fig. 3). The combined action of these enzymes efficiently digests matrix collagen type I of primary tumor spheroids. The aim is to reduce the barrier properties of the collagen-rich ECM and enhance, in vitro, the penetration and cytotoxic effects of chemotherapeutic drugs such as doxorubicin.

Materials

Primary breast tumor spheroids (see Basic Protocol 1)
DPBS (Sigma-Aldrich, cat. no. D8537)

BASIC PROTOCOL 3

Lo Cicero et al.

9 of 19

Collagenase digestion buffer (see recipe)
 FBS (Euroclone, cat. no. ECB3052D)

Class II biosafety cabinet
 Pipettes and sterile filter tips
 Cell culture incubator (37°C, 5% CO₂)
 Ultra-low attachment, U-bottom 96-well plates, sterile (Greiner, cat. no. 650970)
 1.5-ml tubes, sterile
 37°C incubator and/or water bath
 Optical microscope
 Confocal microscope (e.g., Olympus FV10i)

NOTE: Perform all steps with spheroids in 1.5-ml tubes using p1000 tips to minimize mechanical stress.

1. Culture primary breast tumor spheroids (10,000 cells × spheroids) for 6 days in low-attachment 96-well plates under standard conditions (37°C, 5% CO₂) as described in Basic Protocol 1.
2. Transfer each spheroid into a 1.5-ml tube and wash twice with DPBS to remove residual serum proteins.
3. Prepare the collagenase digestion buffer [20 U/ml COLG (class I), 80 U/ml COLH (class II), 2 mM calcium chloride] in HG-DMEM without FBS and supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine.
4. Add 40 µl of collagenase digestion buffer to each of the spheroids and incubate for 75 min at 37°C and 5% CO₂.
5. After incubation, wash spheroids twice with 1 ml of DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine to inactivate collagenases.
6. Proceed with downstream steps, such as drug uptake assays, cell viability, or confocal imaging (from Basic Protocol 2).

BASIC PROTOCOL 4

ASSESSMENT OF DOXORUBICIN UPTAKE IN SPHEROIDS BY CONFOCAL MICROSCOPY

This protocol describes the evaluation of doxorubicin internalization in 3D tumor spheroids by confocal microscopy (Fig. 4). Spheroids, either untreated or pre-treated with recombinant collagenases, are incubated with doxorubicin for different time points to assess the penetration and accumulation of the drug within the 3D structure. The intrinsic fluorescence of doxorubicin allows direct visualization without additional staining. After incubation, spheroids are fixed and processed according to immunocytochemistry procedures, maintaining their structural integrity throughout. Z-stack acquisition by confocal microscopy enables the assessment of drug distribution at various depths of the spheroid.

Materials

Primary tumor spheroids (from Basic Protocol 1)
 Recombinant collagenases (from Basic Protocol 3)
 Doxorubicin stock solution (see recipe)
 HG-DMEM (Sigma-Aldrich, cat. no. D6546)
 DPBS (Sigma-Aldrich, cat. no. D8537)
 Formaldehyde fixative (see recipe)
 DAPI (Sigma-Aldrich, cat. no. D9542)

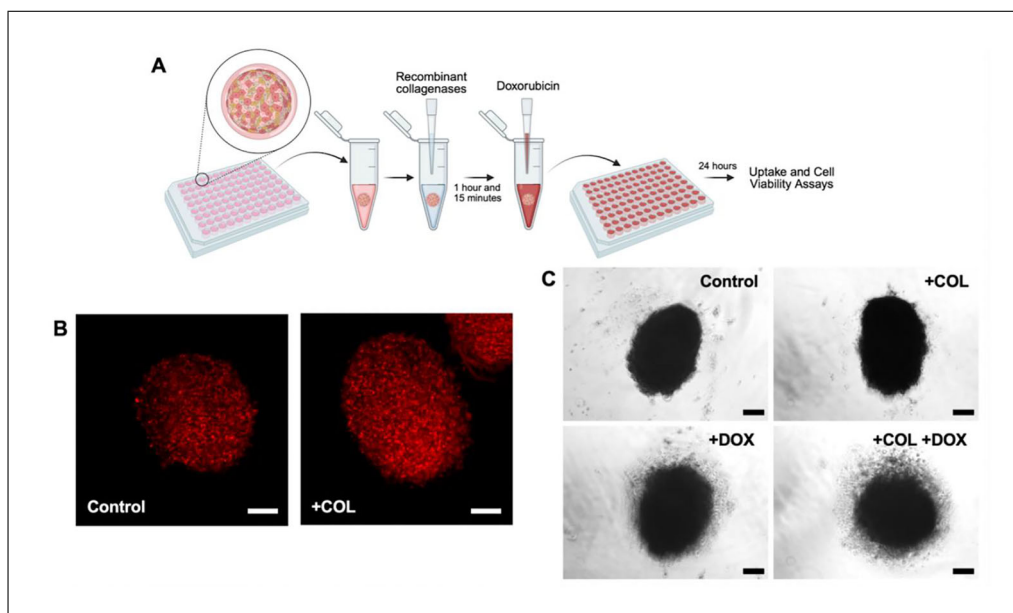


Figure 4 Treatment of spheroid with the anticancer drug doxorubicin. **(A)** Workflow for spheroid treatments and analysis. **(B)** Confocal analysis of doxorubicin (DOX) uptake in control spheroid (Control) and collagenase-treated (+COL). Red, DOX autofluorescence. Scale bar, 100 μm . **(C)** Optical microscopy pictures of control primary tumor spheroid (Control), treated with collagenases (+COL), treated with doxorubicin (+DOX), and with collagenases + doxorubicin (+COL +DOX), after 24 hr of incubation. Scale bar, 100 μm .

Mounting medium: 80% (v/v) glycerol (Sigma-Aldrich, cat. no. 15524), 0.01% (w/v) NaN_3 (Sigma-Aldrich, cat. no. S2002) in 0.2 M Trizma base (Sigma-Aldrich, cat. no. T1503) in HCl (Sigma-Aldrich, cat. no. 258148), pH 7.5

Ultra-low attachment, U-bottom 96-well plates, sterile (Greiner, cat. no. 650970)

1.5-ml tubes, sterile

Pipettes and sterile filter tips

Cell culture incubator, 37°C and 5% CO_2

Rotator

Coverslips and glass slides

Confocal microscope (Olympus FV10i)

ImageJ software (NIH)

1. Collect every single spheroid, treated or untreated with recombinant collagenases as in Basic Protocol 3, from 96-well plates using a p1000 pipette and transfer it into sterile 1.5-ml tubes.
2. Remove the medium and add 100 μl of 20 μM doxorubicin solution (in HG-DMEM culture medium).
Be careful with the medium removal. Try to remove as much as you can to maintain the right concentration of doxorubicin.
3. Replace the spheroid in the culture plate and incubate for 2, 4, 8, or 24 hr at 37°C and 5% CO_2 .
4. After incubation, remove doxorubicin and wash spheroids three times with DPBS to eliminate any drug that was not taken up by the spheroids.

At this point, more than one spheroid can be collected in one tube. Use a p1000 pipette to avoid the disruption of the structure.

Lo Cicero et al.

11 of 19

5. Fix spheroids in 1 ml of 3.7% formaldehyde for 1 hr in slow rotation at room temperature.

Be careful because after the incubation, some spheroids can be placed in the cap of the tube. Gently recover them using a p1000 pipette with DPBS.

6. Wash three times with DPBS.
7. Optionally, stain nuclei with DAPI (1:20,000 in DPBS) for 10 min in slow rotation.
8. Wash three times with DPBS.
9. Mount spheroids on slides by mixing 20 μ l of mounting medium with 20 μ l of DPBS containing spheroids. Apply a coverslip and remove excess liquid.

Use a 1000 μ l pipette to slowly transfer the spheroids onto slides, taking care not to disrupt their structural integrity.

10. Acquire images using a confocal microscope by collecting a z-stack series throughout the spheroid depth.
11. Analyze doxorubicin fluorescence intensity and distribution using ImageJ software.

BASIC PROTOCOL 5

ASSESSMENT OF CELL VIABILITY IN DOXORUBICIN-TREATED SPHEROIDS USING CELLTITER-GLO 3D ASSAY

In this protocol, cell viability in primary tumor spheroids treated with doxorubicin is quantified using the CellTiter-Glo 3D viability assay (Fig. 4). The luminescent signal produced by ATP quantification reflects the metabolic activity of live cells within 3D structures. Spheroids are treated with doxorubicin for various time points, followed by incubation with the CellTiter-Glo 3D reagent, which lyses the spheroids and stabilizes the luminescent signal for measurement with a luminometer.

Materials

Primary tumor spheroids (from Basic Protocol 1)
 Recombinant collagenases (from Basic Protocol 3)
 Doxorubicin stock solution (see recipe)
 HG-DMEM (Sigma-Aldrich, cat. no. D6546)
 DPBS (Sigma-Aldrich, cat. no. D8537)
 CellTiter-Glo 3D reagent (Promega, cat. no. G9683)

Ultra-low attachment, U-bottom 96-well plates, sterile (Greiner, cat. no. 650970)
 Pipettes and sterile filter tips
 1.5-ml tubes, sterile
 Cell culture incubator, 37°C and 5% CO₂
 White 96-well plate (Corning, Turin, Italy, cat. no. CLS3922)
 Plate shaker
 Luminometer (e.g., Synergy HT, BioTek)

1. Collect each spheroid (treated or untreated with collagenases as in Basic Protocol 3) from 96-well plates using a p1000 pipette and transfer it with the medium into sterile 1.5-ml tubes.
2. Remove the medium and incubate spheroids in the 1.5-ml tubes with 20 μ M doxorubicin (in HG-DMEM culture medium) for 24 hr at 37°C.
3. After treatment, recover each spheroid into 1.5-ml tubes and wash the spheroids three times with DPBS to remove residual drug.
4. Resuspend each spheroid in 50 μ l HG-DMEM.

5. Add an equal volume (50 μ l) of CellTiter-Glo 3D reagent directly to each sample, following the manufacturer's instructions.
6. Transfer the spheroids with the 100 μ l solution into a white 96-well plate.
7. Mix gently by shaking the plate for 5 min at 240 rpm to ensure complete lysis of the spheroids.
8. Incubate the plate in the dark at room temperature for 25 min to stabilize the luminescent signal.
9. Record luminescence using a luminometer (e.g., Synergy HT, BioTek).
10. Normalize data to untreated controls to determine relative cell viability.

REAGENTS AND SOLUTIONS

Blocking solution

5 g bovine serum albumin (BSA) (Sigma-Aldrich, cat. no. A7906)
 Bring up to 100 ml with DPBS (Sigma-Aldrich, cat. no. D8537)
 Store up to 1 week at 4°C

Collagenase digestion buffer

20 U/ml collagenase G (COLG) ultrapure recombinant collagenases class I, from
 100 U/ml sterile stock solution in HG-DMEM (Abiel srl, cat. no. 001-001)
 80 U/ml collagenase H (COLH) ultrapure recombinant collagenases class II, from
 500 U/ml sterile stock solution in HG-DMEM (Abiel srl, cat. no. 002-001)
 2 mM calcium chloride (CaCl_2), from 1 M sterile stock solution (Sigma-Aldrich,
 cat. no. 449709)
 HG-DMEM (Sigma-Aldrich, cat. no. D6546)
 100 U/ml penicillin, 100 U/ml streptomycin (Euroclone Celbar, cat. no. ECB3001)
 2 mM L-glutamine (Euroclone Celbar, cat. no. ECB3000D)
 Prepare fresh, keep on ice, and use immediately

Complete HG-DMEM

HG-DMEM (Sigma-Aldrich, cat. no. D6546)
 10% (v/v) FBS (Euroclone, cat. no. ECB3052D)
 100 U/ml penicillin, 100 U/ml streptomycin (Euroclone Celbar, cat. no. ECB3001)
 2 mM L-glutamine (Euroclone Celbar, cat. no. ECB3000D)
 Store up to 1 month at 4°C

Digestion buffer

HG-DMEM (Sigma-Aldrich, cat. no. D6546)
 200 U/ml penicillin, 200 U/ml streptomycin (Euroclone Celbar, cat. no. ECB3001)
 2 mM L-glutamine (Euroclone Celbar, cat. no. ECB3000D)
 106.5 U/g collagenase G (COLG) ultrapure recombinant collagenases class I, from
 100 U/ml sterile stock solution in HG-DMEM (Abiel srl, cat. no. 001-001)
 453.2 U/g collagenase H (COLH) ultrapure recombinant collagenases class II,
 from 500 U/ml sterile stock solution in HG-DMEM (Abiel srl, cat. no. 002-001)
 266.4 μ g/ml thermolysin from *Geobacillus stearothermophilus* (sterile stock
 solution = 1 mg/ml) (Sigma-Aldrich, cat. no. T7902)
 2 mM calcium chloride (CaCl_2) (sterile stock solution = 1 M) (Sigma-Aldrich, cat.
 no. 449709)
 Prepare fresh, keep on ice, and use immediately

Doxorubicin stock solution, 2 mg/ml

Prepare 2 mg/ml doxorubicin hydrochloride (Sigma-Aldrich, cat. no. D2975000) in sterile water. Store up to 2 weeks at 4°C protected from light.

Formaldehyde fixative, 3.7%

Dilute 37% formaldehyde solution (Sigma-Aldrich, cat. no. 252549) 1:10 in DPBS (Sigma-Aldrich, cat. no. D8537). Store up to 1 week at 4°C.

COMMENTARY

Background Information

Limited penetration of chemotherapeutic agents into solid tumors remains a major obstacle to effective cancer treatment and a key contributor to therapeutic failure (Minchinton & Tannock, 2006). This limitation arises not only from cellular drug resistance mechanisms, but also from the complex architecture of the tumor microenvironment, in which the extracellular matrix (ECM) plays a central role (Lo Buglio et al., 2024). The ECM provides structural support to tumor tissue while simultaneously acting as a physical and biochemical barrier that restricts drug diffusion, alters cellular signaling, and modulates therapeutic response (Henke et al., 2020; Huang et al., 2021b; Jurj et al., 2022).

Among ECM components, fibrillar collagens are particularly abundant in many solid tumors and are strongly associated with increased tissue stiffness, reduced vascular perfusion, and impaired drug delivery (Bourgot et al., 2020; Fuller & Eisinger-Mathason, 2022). Dense collagen networks limit the transport of small-molecule chemotherapeutics such as doxorubicin, leading to heterogeneous intratumoral drug distribution and reduced exposure of cells located in poorly perfused or deeply embedded tumor regions (Fu et al., 2025). Consequently, cancer cells within the tumor core may survive treatment and contribute to disease relapse and chemoresistance.

Two-dimensional (2D) cell culture fails to capture these microenvironmental barriers, as it lacks the endogenous ECM deposition characteristic of solid tumors (Kapałczyńska et al., 2016). In contrast, three-dimensional (3D) tumor spheroids reproduce key structural and functional features of in vivo tumors, making them particularly well suited for investigating how ECM composition and organization influence drug penetration, accumulation, and cytotoxicity under controlled experimental conditions (Druzhkova et al., 2022; Y. Li & Kumacheva, 2018; Longati et al., 2013).

The protocols described here leverage primary tumor-derived spheroids, which pre-

serve tumor-specific cellular heterogeneity and matrix composition more faithfully than spheroids generated from established cancer cell lines (Lo Cicero et al., 2025). Primary tumor cells deposit their own ECM, generating a collagen-rich microenvironment that closely mimics diffusion barriers encountered in vivo (Zhang & Zhang, 2025). This feature is essential for evaluating microenvironment-driven modulation of drug response and toxicity.

To directly interrogate the role of collagen in limiting drug accessibility, controlled enzymatic remodeling of the endogenous ECM is performed using ultrapure recombinant collagenases (Salamone et al., 2014; Volpe et al., 2016), combining two classes of these enzymes: Class I and Class II. Class I (COLG) recognizes the native collagen, acting on the 3D-helix region, while Class II (COLH) has a higher activity against the linear collagen region (Breite et al., 2011). This approach allows selective reduction of collagen density while maintaining spheroid architecture and cell viability, thereby enabling mechanistic investigation of ECM-dependent diffusion barriers. Doxorubicin is employed as a model chemotherapeutic agent due to its widespread clinical use, well-defined cytotoxic mechanism, and intrinsic fluorescence, which permits direct visualization of its spatial distribution within intact 3D structures (Tacar et al., 2013). Notably, cancer cells cultured in 3D ECM-based models show reduced sensitivity to doxorubicin compared with 2D monolayer cultures, a phenomenon largely driven by cell–ECM interactions and associated pro-survival signaling pathways (Lovitt et al., 2018).

By integrating ECM remodeling with confocal imaging-based drug uptake analysis and quantitative viability assays, this workflow provides a physiologically relevant and experimentally tractable platform to study how the tumor microenvironment influences drug penetration and cytotoxic efficacy. This system bridges the gap between simplified in vitro assays and in vivo tumor models and supports the development and evaluation of strategies

aimed at overcoming ECM-mediated chemoresistance (Lo Cicero et al., 2025).

Critical Parameters

Several experimental variables critically influence the reliability and reproducibility of this workflow and must be carefully controlled.

Spheroid formation and uniformity

Consistent spheroid size and morphology are essential for comparative analyses. Variability in initial cell seeding density or aggregation efficiency can alter ECM deposition, diffusion distances, and drug response. Spheroids should be generated under standardized low-attachment conditions and used at comparable time points.

Enzymatic ECM degradation

The concentration and ratio of recombinant collagenases, as well as incubation time, determine the extent of ECM digestion. The combined use of collagenase class I (COLG) and class II (COLH) enables efficient degradation of fibrillar collagens while preserving spheroid cohesion. Over-digestion may lead to spheroid disintegration, whereas insufficient digestion may not significantly enhance drug penetration. Thorough washing with serum-containing medium is required to fully inactivate residual enzyme activity.

Drug treatment conditions

Doxorubicin concentration and exposure duration must be optimized. Collagenase-treated spheroids may require lower drug concentrations to achieve cytotoxicity comparable to untreated controls, emphasizing the importance of dose normalization across conditions.

Imaging and analysis parameters

Confocal microscopy settings, including laser intensity, detector gain, and z-step size, should be kept constant across samples to allow quantitative comparison of fluorescence intensity. Z-stack acquisition should cover the full spheroid depth to accurately assess spatial drug distribution.

Viability assay performance

Complete spheroid lysis is essential for accurate ATP quantification in luminescent viability assays. Inadequate mixing or insufficient incubation with the CellTiter-Glo 3D reagent can result in underestimation of cytotoxic effects, particularly in larger spheroids.

Troubleshooting

For possible problems and solutions for executing these protocols, please see Table 1.

Understanding Results

Successful implementation of these protocols should result in the reproducible formation of compact, spherical primary tumor spheroids with smooth, well-defined borders and minimal spontaneous disaggregation. At early culture stages, spheroids are expected to exhibit a dense peripheral region and a compact core, reflecting effective cell–cell interactions and deposition of endogenous ECM (Fig. 2). Immunofluorescence analysis should confirm abundant collagen type I (Fig. 3) throughout the spheroid, indicating the establishment of a collagen-rich microenvironment capable of imposing diffusion constraints similar to those observed in solid tumors in vivo (Bonans et al., 2014).

Following enzymatic treatment with recombinant collagenases, confocal microscopy should reveal a pronounced reduction or near-complete loss of collagen type I staining across the spheroid (Fig. 3). Importantly, effective ECM degradation is characterized by preservation of overall spheroid architecture, with maintained cohesion and absence of extensive fragmentation. Partial disaggregation or loss of spheroid integrity indicates excessive enzymatic digestion, necessitating adjustment of collagenase concentration or incubation time.

In doxorubicin uptake experiments, untreated spheroids typically display restricted drug penetration, with strong fluorescence localized to outer layers and minimal signal in the core, even after prolonged incubation (Fig. 4). This pattern reflects ECM-mediated diffusion barriers and resembles the heterogeneous drug distribution observed in poorly perfused tumor tissues (Gong et al., 2025). In contrast, collagenase-pretreated spheroids should exhibit enhanced and more uniform doxorubicin fluorescence across multiple z-stacks, indicating increased penetration depth and improved accessibility to the spheroid core. This effect is expected to be time-dependent, with progressive accumulation at longer exposure periods. Quantitative analysis of confocal z-stacks should demonstrate significantly increased mean fluorescence intensity in inner spheroid regions after collagenase treatment compared with untreated controls. Spatial fluorescence profiling confirms that the observed increase represents enhanced intratumoral distribution rather than nonspecific surface binding.

Table 1 Troubleshooting Guide for the Isolation of Tumor Primary Cells, Generation and Enzymatic Degradation of the Endogenous Matrix Collagen of 3D Spheroids and Fluorescence Analysis

Problem	Possible cause	Solution
Contamination of cells of adjacent tissue	The isolated tumor mass contains pieces of other tissues	Clean the tumor mass well, take care to remove all the other tissue that can appear with a different color and consistency
Obtained dead cells	The treatment of the tumor mass with 70% ethanol is too long	Take care to treat the isolated tumor mass for no longer than 2-3 min with 70% ethanol
	The treatment with collagenase is too long	After the addition of collagenases, control the tumor mass under the optical microscope and, if the tissue is totally digested, stop the digestion
Low cell yield	Poor cell separation technique	Save all supernatants; repeat cell counts for all supernatants
Poor or inconsistent spheroid formation	Incorrect seeding density	Accurately count cells and seed exactly 1×10^4 cells per well, and resuspend the cell suspension immediately before seeding
Incomplete collagen degradation	Low cell viability or excessive passaging	Use cells with $\geq 90\%$ viability and avoid excessive passage numbers
Spheroid disintegration	Over-digestion due to excessive incubation	Reduce incubation time and handle spheroids gently
Residual collagenase activity affecting downstream assays	Inadequate washing post-digestion	Wash spheroids at least twice with medium containing 10% FBS
Collagenases inactivation during the spheroid's incubation	Interaction with other drugs	Incubate the spheroids with collagenases before, and only then proceed with the other treatments
Loss or damage of spheroids during washes	Aspiration too strong or spheroids adhering to the tube walls/cap	Use a p1000 pipette and carefully check both tube bottom and the cap after each wash
High background fluorescence	Incomplete washing or excessive antibody concentration	Increase the number or duration of DPBS washes; optimize blocking time (1-2 hr)
Flattened or disrupted spheroids on a slide	Mechanical stress during mounting	Optionally use imaging chambers instead of standard slides if your spheroids are $>400 \mu\text{m}$
Weak or no doxorubicin fluorescence	Low uptake due to dense or bigger spheroid	Increase incubation time or concentration slightly (up to $30 \mu\text{M}$), depending on spheroid dimension; alternatively, reduce spheroid size ($<400 \mu\text{m}$ diameter); consider collagenase pre-treatment to enhance penetration
Heterogeneous signal (dark core)	Limited diffusion of doxorubicin into the spheroid center	Extend incubation and ensure rotation during drug incubation
Low or inconsistent luminescence signal	Incomplete lysis of spheroids or loss of the spheroid during pipetting	Ensure equal reagent volumes (1:1 ratio); use replicates to minimize variability
Overly strong signal (saturation)	Spheroids are too large and contain an excessive cell number	Reduce spheroid size

Cell viability assessment using the CellTiter-Glo 3D assay should align with imaging-based observations (Fig. 4). Spheroids exposed only to doxorubicin generally show just a modest reduction in ATP levels compared to the untreated controls, reflecting limited drug access to metabolically active cells in the spheroid core. In contrast, collagenase-treated spheroids exposed to doxorubicin should display a more pronounced decrease in ATP levels, indicating enhanced cytotoxic efficacy due to improved drug penetration. Collagenase treatment alone is not expected to significantly affect ATP levels, confirming that enzymatic ECM remodeling does not induce overt cytotoxicity under optimized conditions.

Overall, these results demonstrate that selective enzymatic remodeling of the collagen-rich ECM effectively reduces diffusion barriers in 3D tumor spheroids, resulting in increased intratumoral accumulation of doxorubicin and potentiation of its cytotoxic effects. Deviations from these expected outcomes, such as minimal enhancement of drug penetration or extensive spheroid disintegration, suggest suboptimal spheroid quality, incomplete ECM degradation, or technical issues during imaging or viability measurements. This integrated analysis provides a robust framework for understanding how microenvironmental factors modulate drug distribution and cytotoxicity in physiologically relevant 3D tumor models.

Time Considerations

Below lists various procedures detailed in this article and the time required for each:

Induction of breast cancer, 6 months
Isolation of tumor primary cells, ~4 hr
Spheroid formation, 5 to 6 days
ECM digestion, 1.5 hr
Immunostaining, 2 days
Doxorubicin penetration assay, 2 to 24 hr
CellTiter-Glo 3D, ~1 hr.

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Author Contributions

Alessandra Lo Cicero: Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft; writing—review and editing. **Gabriele Lo Buglio:** Data curation; formal analysis; investigation; methodology; writing—original draft; writing—review and editing. **Simona Campora:** Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft; writing—review and editing. **Giulio Gherzi:** Conceptualization; funding acquisition; methodology; project administration; resources; supervision; validation; writing—original draft; writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

No underlying data are available for this article, since no datasets were generated or analyzed during this study.

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