PrimeSurface
Anticancer Drug screening by:

Spheroid Cell Culture

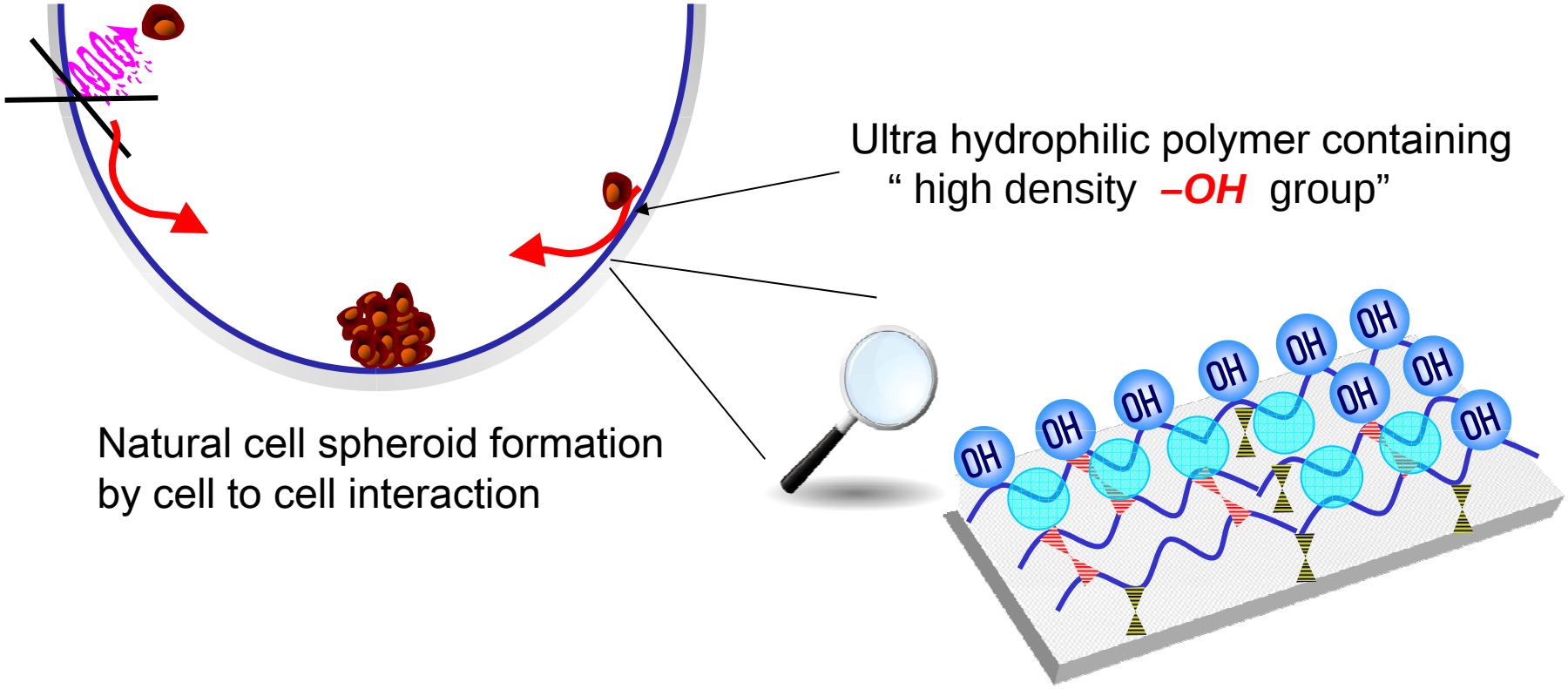
Sumitomo Bakelite Co., Ltd.

S-BIO Business Division

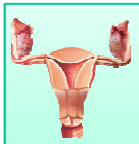
Feature of PrimeSurface

- Complete Cell Non-adhesion Surface
- Uniform Spheroid Formation
- A Variety of Well Shapes

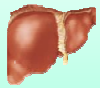
Principle of Spontaneous Multicellular Tumor Spheroid Formation with PrimeSurface



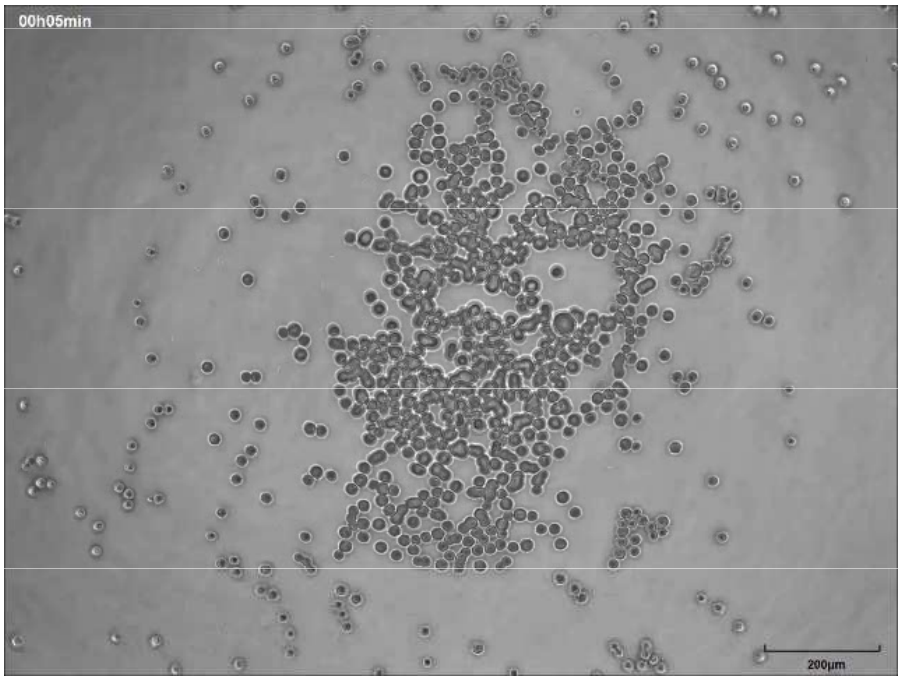
Time-Lapse of Spheroid Formation

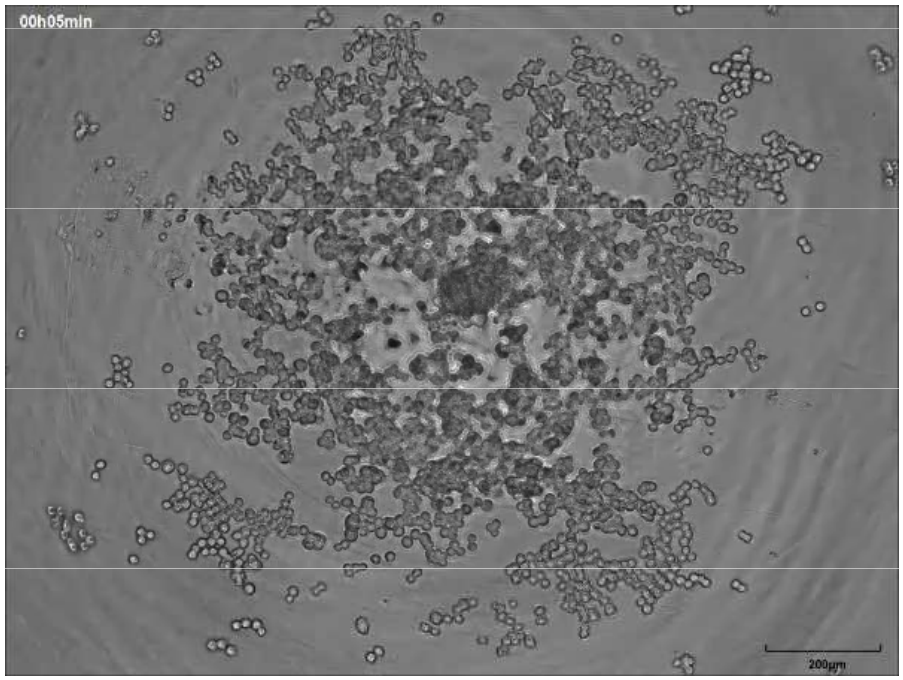


96 well plate
HeLa cell
1,000 cells/well/100μL
MEM＋10%FBS

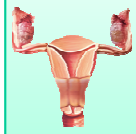


384 well plate
HepG2 cell
1,000 cells/well/50μL
DMEM（Low Glucose ）＋10%FBS

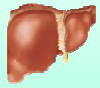




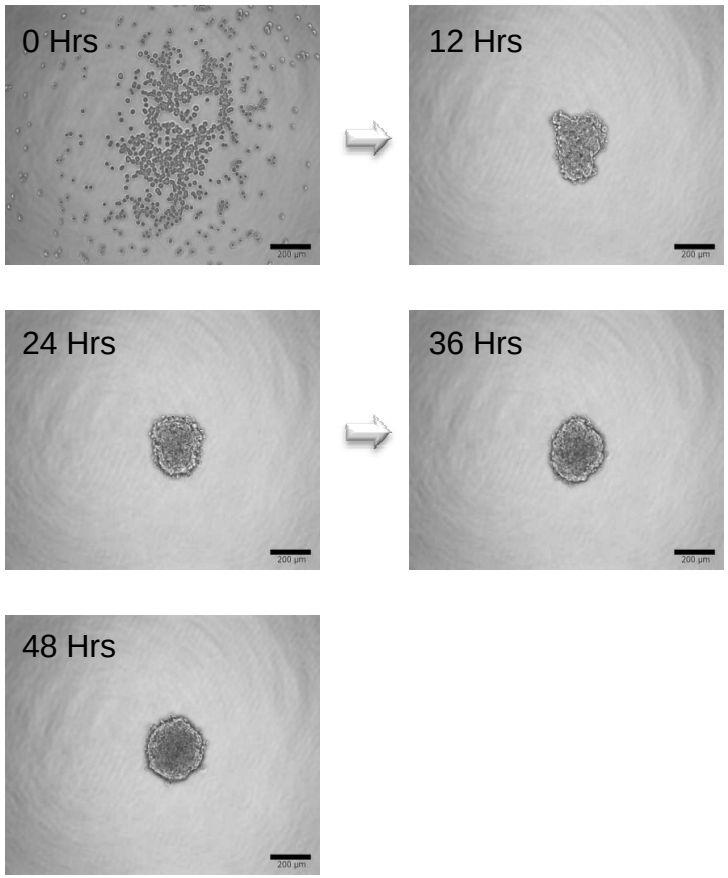
Time Course Change of Spheroid Formation

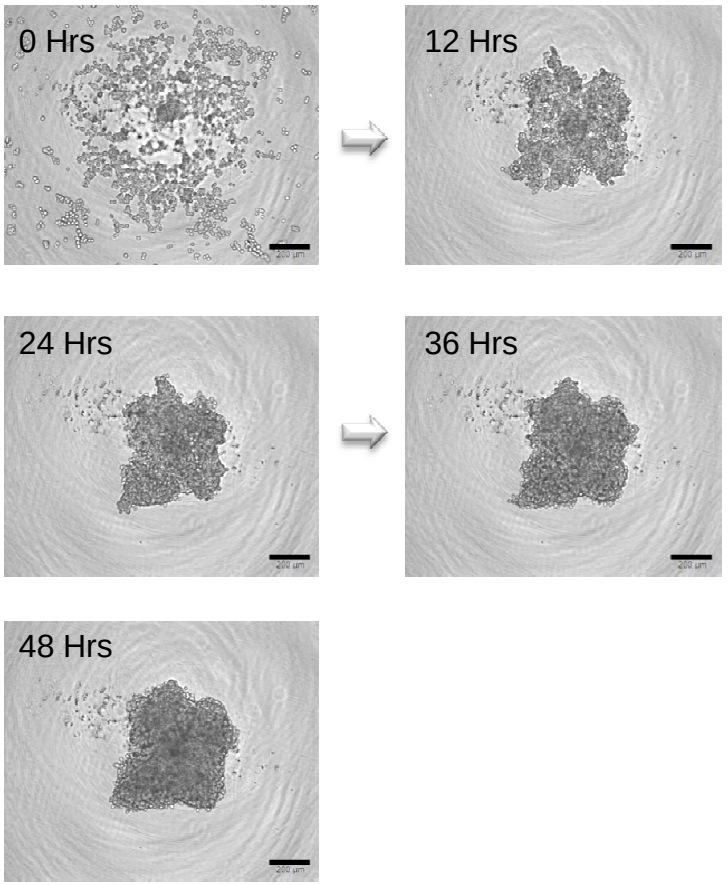


96 well plate
HeLa cell
1,000 cells/well/100μL
MEM＋10%FBS



384 well plate
HepG2 cell
1,000 cells/well/50μL
DMEM（Low Glucose）＋10%FBS



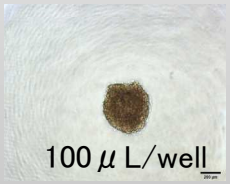


New Lineup! PrimeSurface 384 Multiwell Plate

New Lineup

96 well type

Max volume 300µL



Throughput Up

HTS by Robot Operation

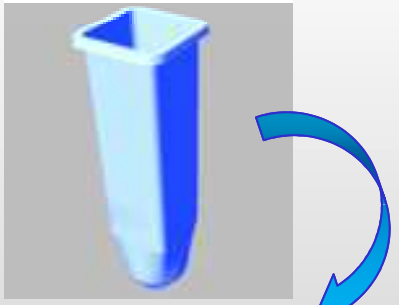
Easy Operation

Medium Volume Reduction

384 well type

PrimeSurface 384 round well (Clear & White)

Max volume *106µL* !

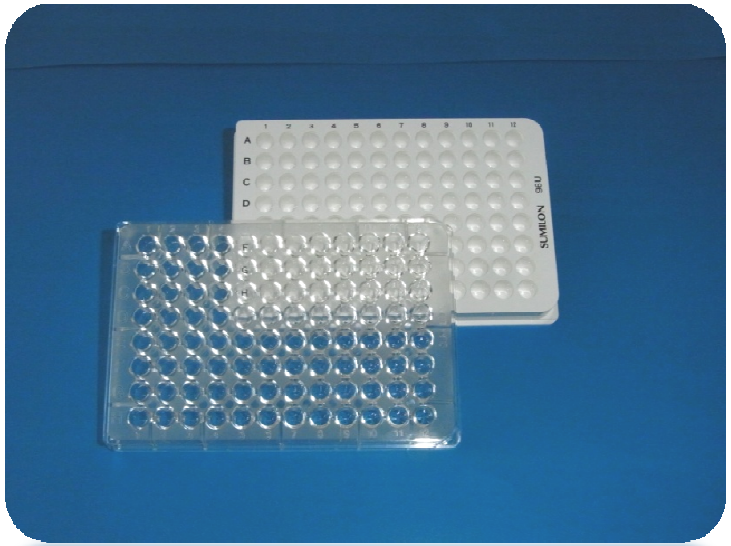


Just dispense cells

【 Experimental conditions 】

Cell type ：HepG2, Culture Medium: DMEM low Glc. + 10%FCS , Seeding Density: both 1,000cell /well , Culture Period: 3 Days

Lineups of PrimeSurface 96 Well Type

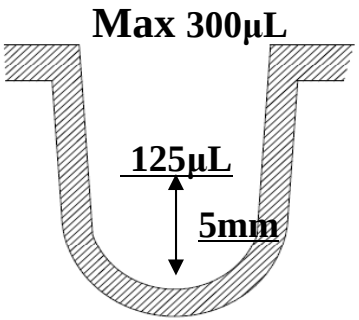


Select the well shape and finish

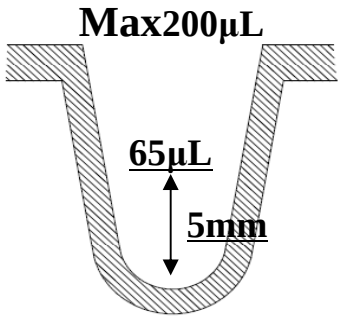
according to your cell properties

(such as cell aggregation ability).

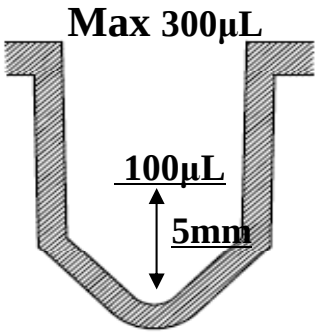
MS-9096U（96 well clear）
MS-9096W（96 well white）



MS-9096M（96 well clear）

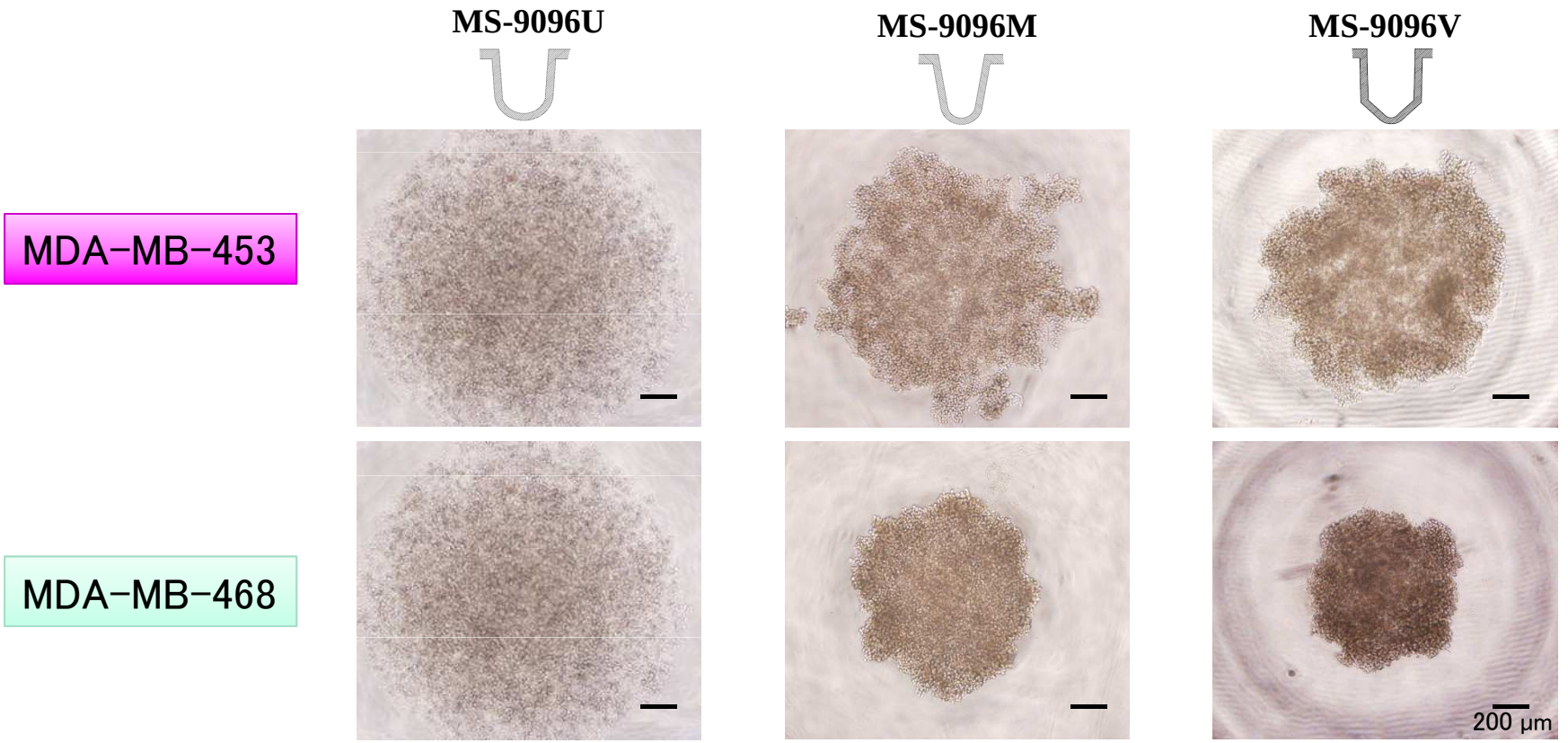


MS-9096V（96 well clear）



Effect of Well Bottom Shape on Spheroid Formation

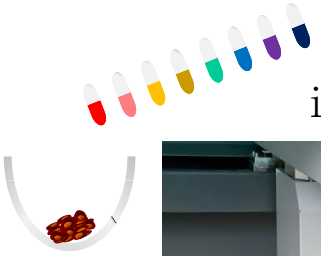
The *steep shaped bottom* successfully supported
the weak aggregation ability of below cancer cells.



Seeding Density: 2x10^3 cells/well, Culture Medium: RPMI + 10%FBS, Incubation: 37℃, 5%CO_2, Culture Period: 7 Days
MDA-MB-453, MDA-MB-468: human breast cancer

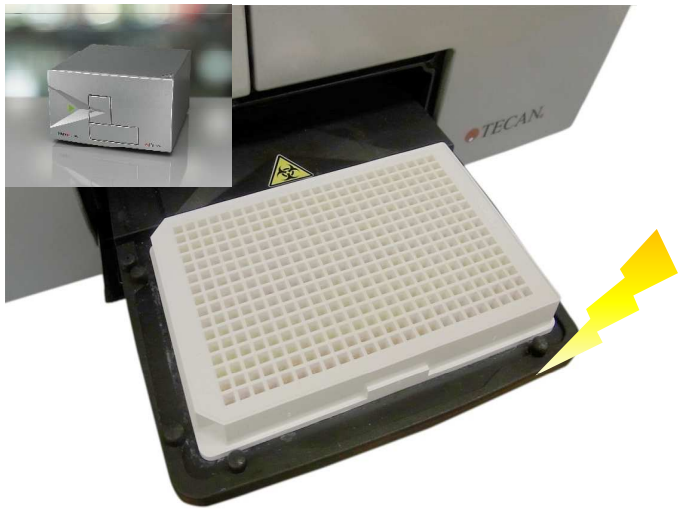
Data are provided by Nishio Lab., Dept. of Genome Bio. Kinki Univ. Faculty of Medicine

PrimeSurface White Plate Enables One-Stop Assay



i ）Addition of drug candidates into the well

ii ）Addition of analytical reagent



iii ）Chemiluminescence measurement

Robot Dispenser: Freedom EVO® , Plate Reader: Infinite® 200 PRO
Photos are supplied Tecan Japan Co., Ltd

Both Spheroid Cell Culture and Chemiluminescence Measurement can be Performed in the Same Well

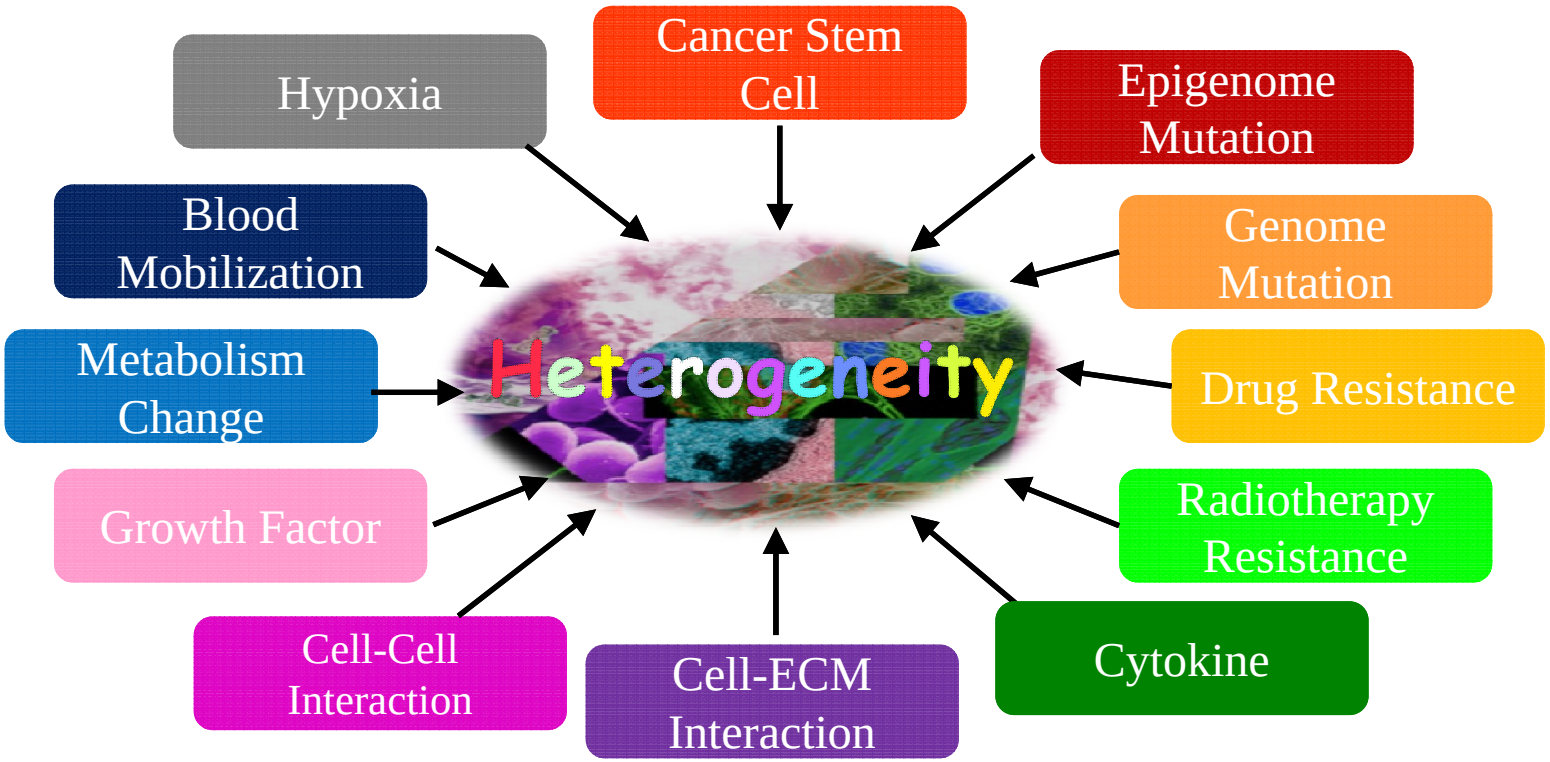
Reduction of Assay Steps &Time, High Speed !

Significances of Multicellular Spheroid Culture for Drug Research & Development

Spheroid Cell Culture Mimics *in vivo* Heterogeneity Surrounding Tumor Cells

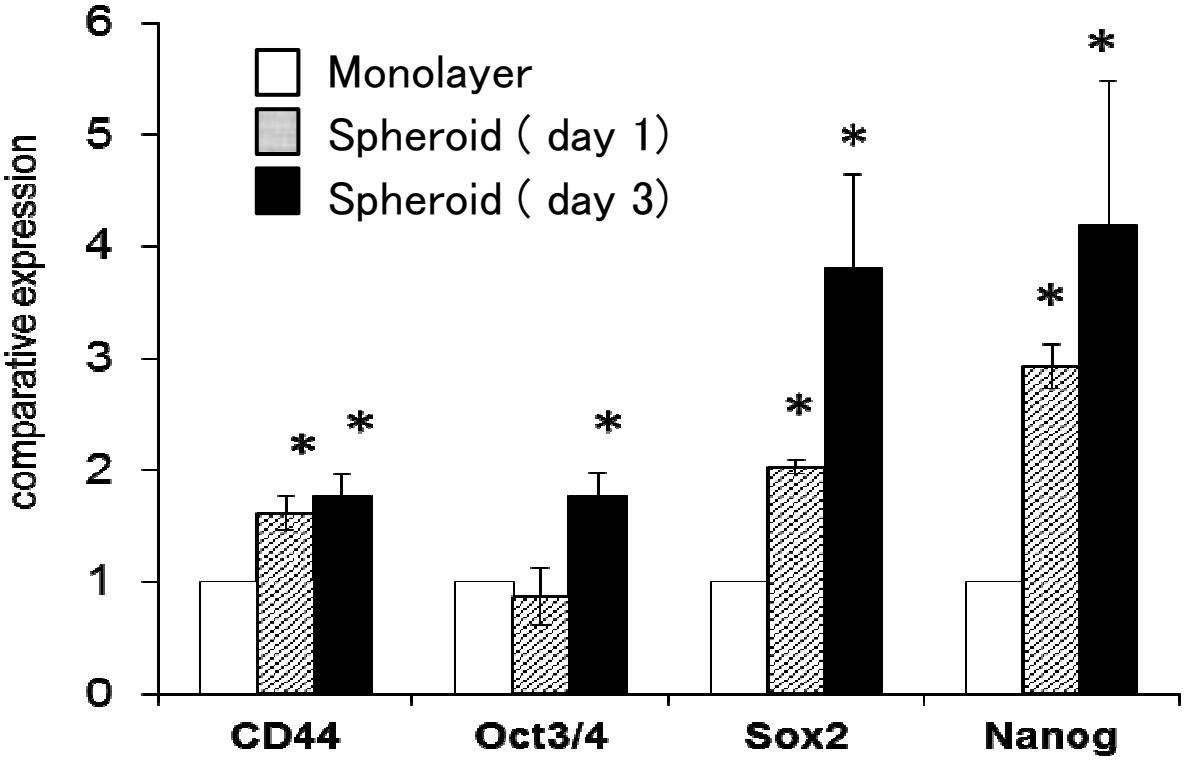
Reconstruction of
Microenviroment and *Heterogeneity*

- Spheroid Culture ○
- Monolayer Culture ×



[Reference] Fujita Y., et al., Experimental Biology (2013), *l.31* (1), 2-7

Genetic Characters of MCTS



Comparison of gene expression profiles between Monolayer and Spheroid Cultured Cancer Cells (Cells: MDA-MB-231, Plate: PrimeSurface® MS-9096U)

Significant increase in genes expression, such as CD44, Oct3/4, Sox2, Nanog, were observed in the spheroid cultured cells.

The above Data are provided by Nishio Lab., Dept. of Genome Bio. Kinki Univ. Faculty of Medicine.

Comparison of Anticancer Drug Efficacy between Monolayer Culture and Spheroid Culture

【Experiment Ⅰ】

Data are provided by Nishio Lab., Dept. of Genome Bio. Kinki Univ. Faculty of Medicine.

【Experiment Ⅱ】

Other data are obtained in our company

Experiment Ⅰ：
Evaluation Examples of Anticancer Drug Efficacy

【 Culture Methods 】

Monolayer vs Spheroid（PrimeSurface）

【 Cells 】

MDA-MB-231（◆）， BT-549（■）， MCF-7（▲）

【 Anticancer Drugs 】

Cisplatin（CDDP），5-Fluorouracil（5-FU），Docetaxel（DOC），and SN-38

【 Evaluation Items 】

- Viability assay by live cell protease activity measurement
- Live/Dead immunofluolor double staining

Experiment Ⅰ

Spheroid Size Change with Time in PrimeSurface 96 Multiwell Plate

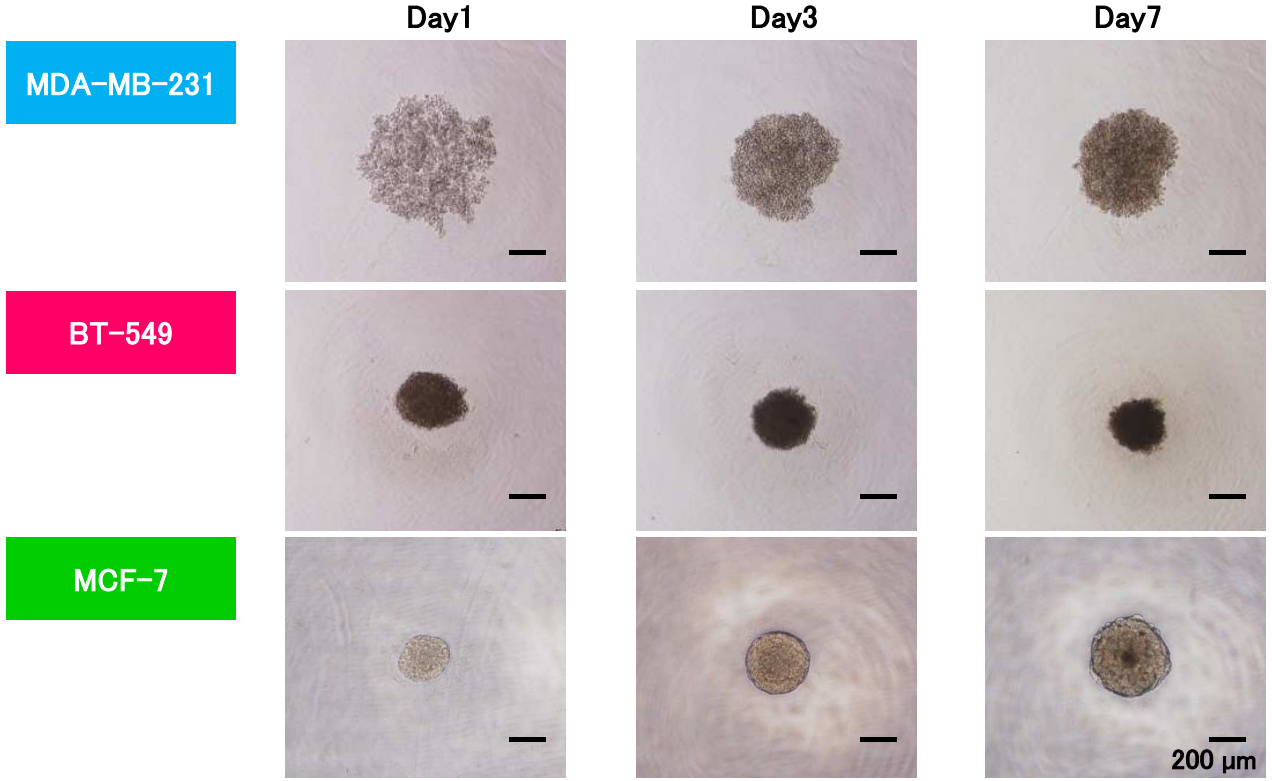
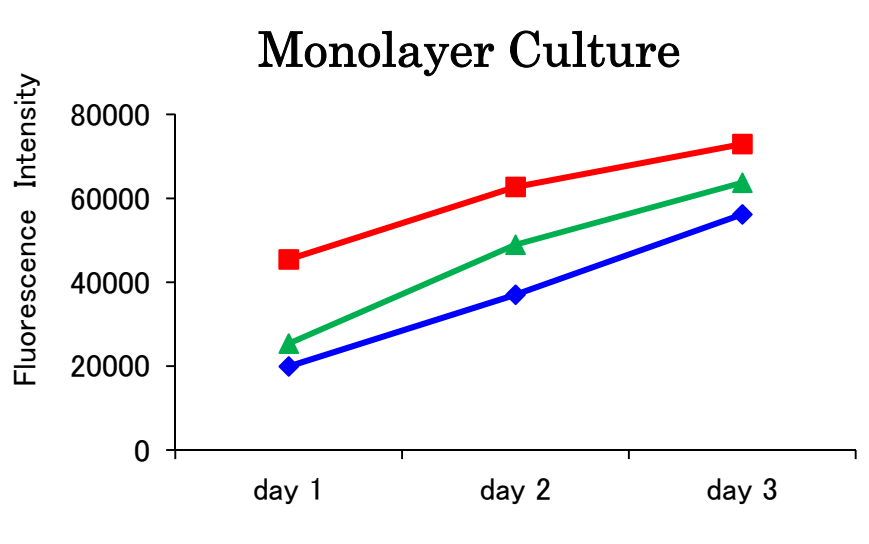
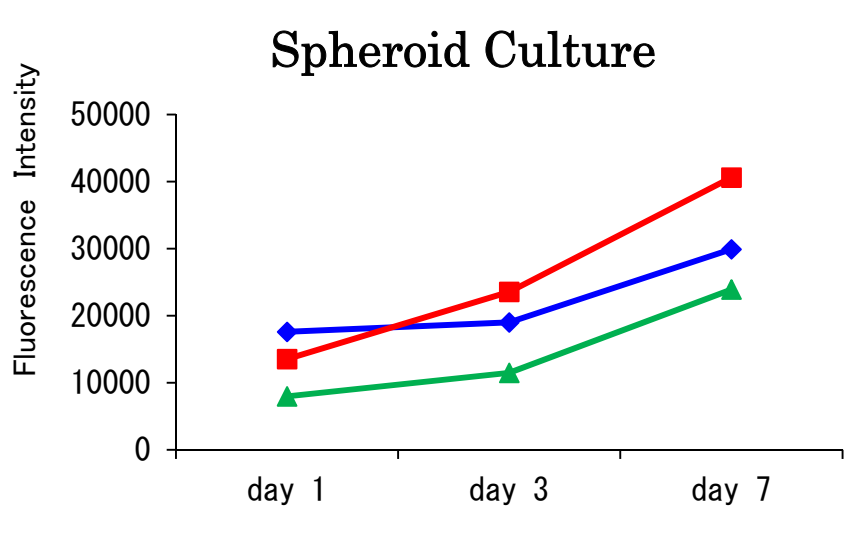


Plate	: PrimeSurface® MS-9096U
Seeding Density	: 2x10^3 cells/well
Culture Medium	: RPMI + 10%FBS, 37℃, 5%CO_2
Culture Period	: 7Days
Cells	: MDA-MB-231, BT-549, MCF-7 (Human Breast Cancer)

Uniform spheroids were maintained up to Day 7

Experiment Ⅰ
Evaluation of Cell Proliferation





◆:MDA-MB-231, ■: BT-549, ▲: MCF-7

【 Materials & Methods 】

① Seed cells into both conventional 96 multiwell plate MS-8096F (Monolayer Culture) and PrimeSurface® MS-9096U plate (Spheroid Culture) ($2x10^3$ cells/100μL/well).

② Culture cells at 37℃, 5%CO_2.

③ Add a 100 μL aliquot of CellTiter-Fluor™ Cell Viability Assay reagent into each well at Day 1, 2 and 3(Monolayer Culture) or Day 1,3 and 7(Spheroid Culture).

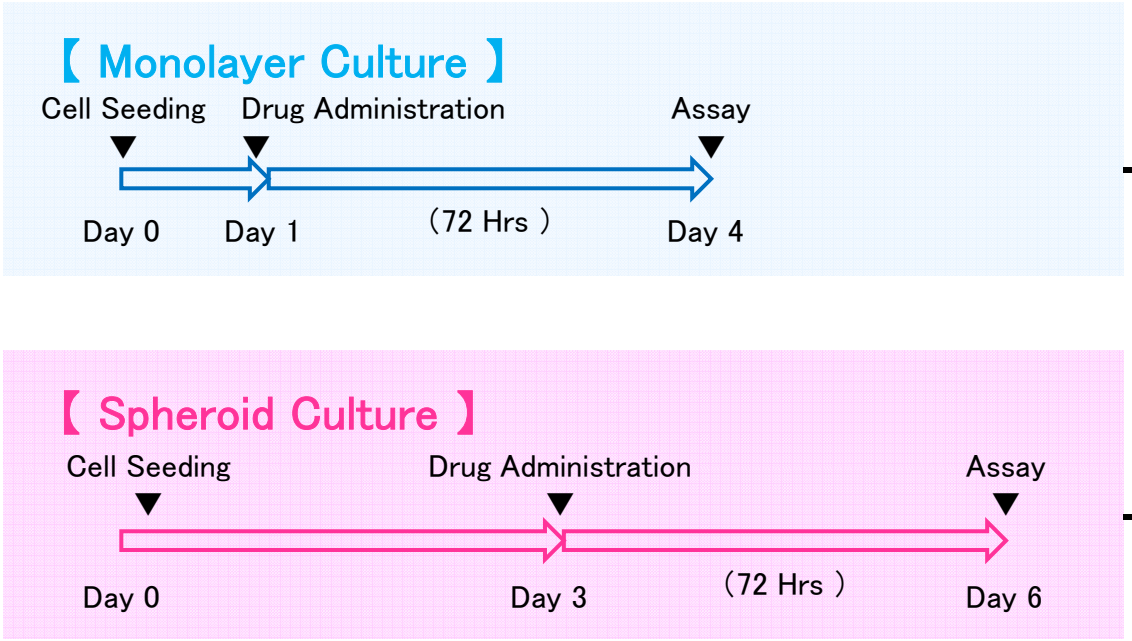
④ Incubate cells at 5% CO_2, 37℃ for 1 Hr under dark.

⑤ Transfer the whole reaction mixture into the black plate well.

⑥ Measure fluorescence intensity at 400 nm/505 nm (Ex/Em).

Satisfactory cell proliferations were observed in PimeSurface

Experiment Ⅰ
Cell Seeding , Drug Administrations and Assay Conditions



Drug administration periods are 72Hrs for both monolayer and spheroid culture in common.

【 Cell Seeding 】

Seed cells into wells into both the conventional into conventional 96 multiwell plate and PrimeSurfaceMS-9096U plate ($2x10^3$cells/100μL/well).

【 Drug Administration 】

① Aspirate and discard a 50 μL aliquot of culture medium at Day 1(Monolayer Culture) or Day3 (Spheroid Culture).
② Add a 50μL aliquot of anticancer drug solution.

Experiment Ⅰ
Methods of Viability Assay by Live Cell Protease Activity Measurement and Live/Dead Immunofluolor Double Staining.

【 Viability Assay by Live Cell Protease Activity Measurement 】

(Promega Co., CellTiter-Fluor™ Cell Viability Assay Kit)

① Add CellTiter-Fluor™ Cell Viability Assay reagent into each well (100 μL/well) 72Hrs after anticancer drug administration.

② Incubate cells at 5% CO_2, 37℃ for 1 Hr under dark.

③ Transfer the whole reaction mixture into black plate well.

④ Measure fluorescence intensity at 400 nm/505 nm(Ex/Em).

【 Live/Dead Immunofluolor Double Staining of Spheroids 】

(Lonza Co., Live / Dead® Viability/Cytotoxicity Assay Kit)

① Aspirate the culture medium carefully not to aspirate the spheroids.

② Add a 100μL aliquot of PBS(−) into every wells gently.

③ Aspirate and discard PBS(−) carefully not to aspirate the spheroid.

④ Repeat ③—④ steps twice.

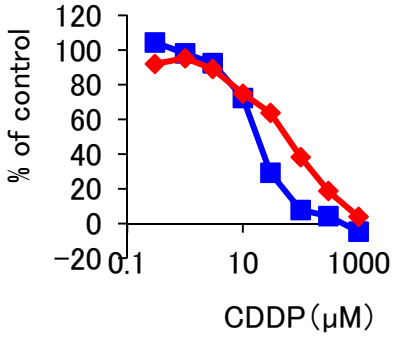
⑤ Add a 25μL aliquot of 4μM Calcein AM / 8μM EthD-1 PBS(−) solution into every well.

⑥ Incubate cells at 5% CO_2, 37℃ for 30 minutes under dark.

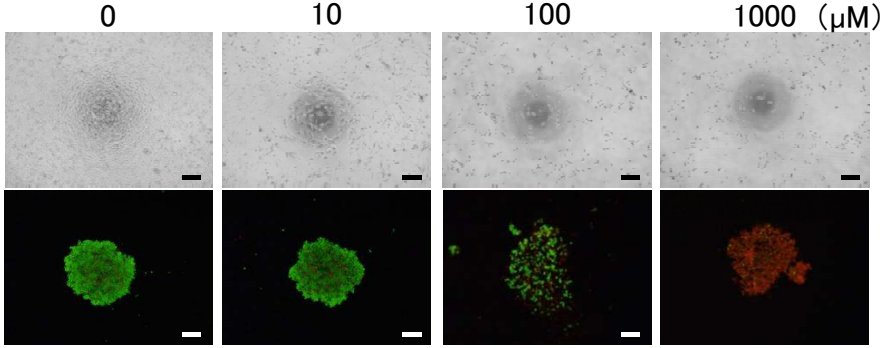
⑦ Observe Live Cells (Ex/Em ～495nm/～515nm:Green) and Dead Cells (Ex/Em ～495nm/～635nm:Red) with fluorescence microscope.

Experiment I
Result Example 1: Cisplatin (CDDP)

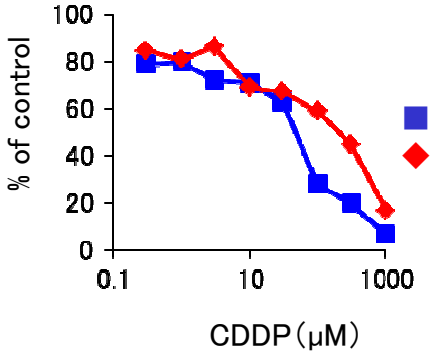
MDA-MB-231



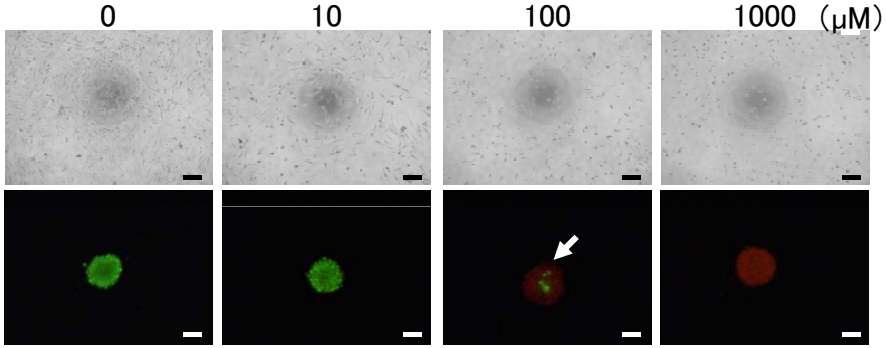
■ : Monolayer
◆ : Spheroid



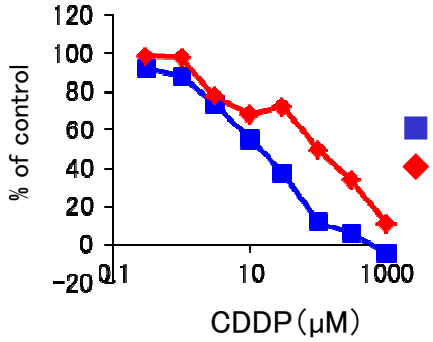
BT-549



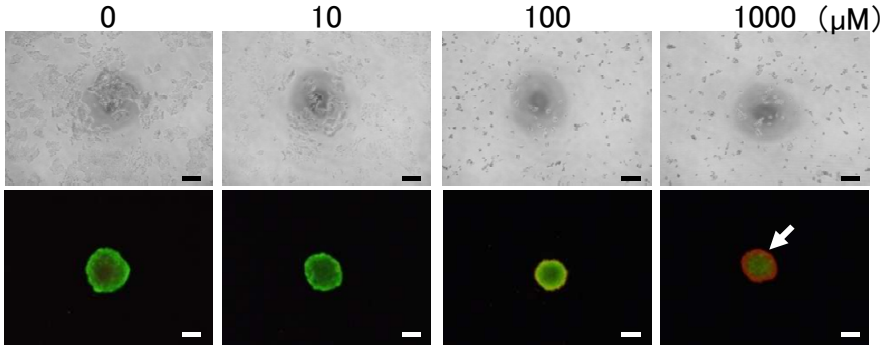
■ : Monolayer
◆ : Spheroid



MCF-7



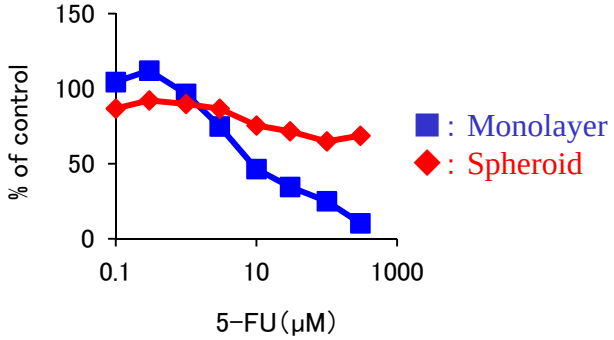
■ : Monolayer
◆ : Spheroid



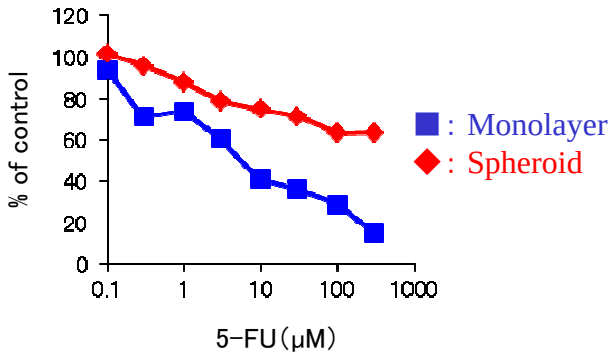
Scale bars : 200 μm
Upper : a phase-contrast microscope images
Lower : Live/Dead immunofluolor double staining images
Arrows (↙) : Cells in the peripheral regions are more susceptible to anticancer drugs than those in center region

Experiment I
Result Example 2: 5-Fluorouracil（5-FU）

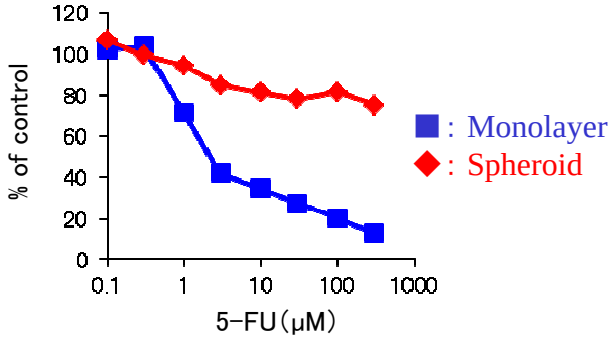
MDA-MB-231

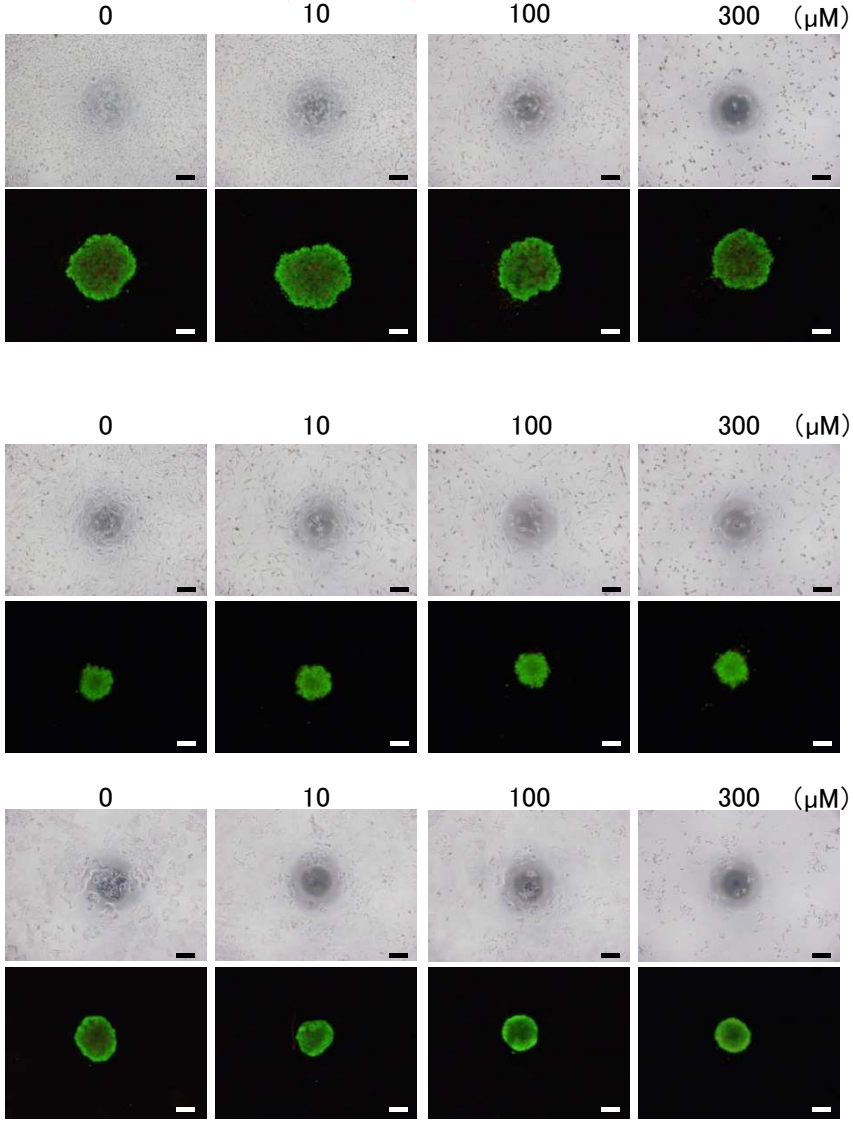


BT-549



MCF-7

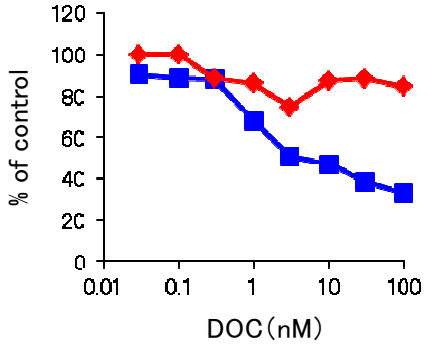




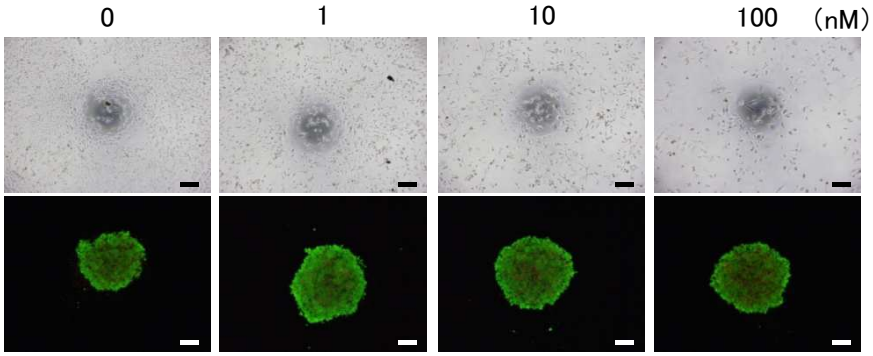
Scale bars : 200 μm
Upper : a phase-contrast microscope images
Lower : Live/Dead immunofluolor double staining images

Experiment I
Result Example 3: Docetaxel (DOC)

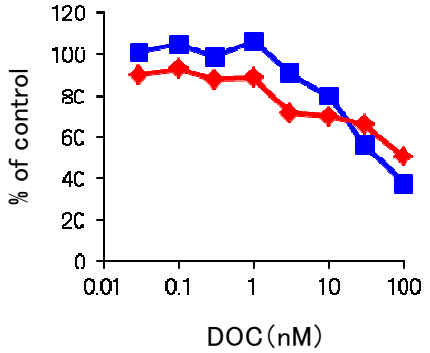
MDA-MB-231



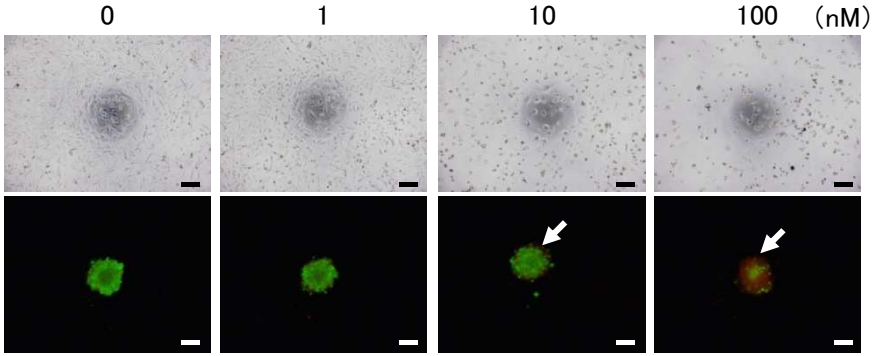
■ : Monolayer
◆ : Spheroid



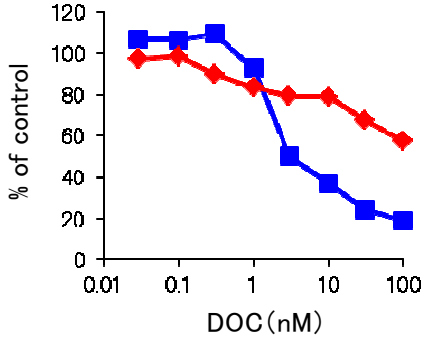
BT-549



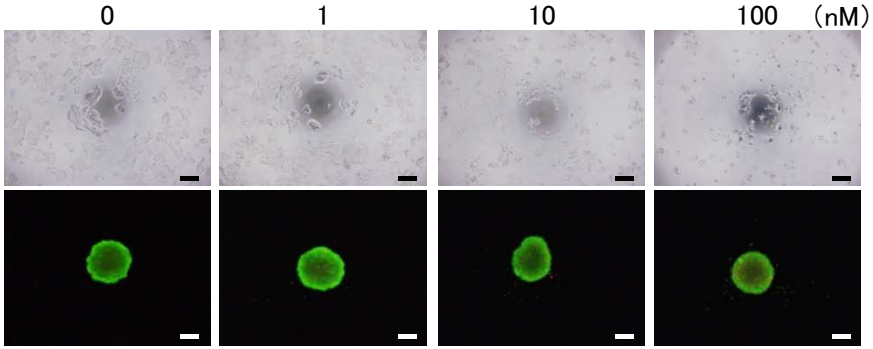
■ : Monolayer
◆ : Spheroid



MCF-7



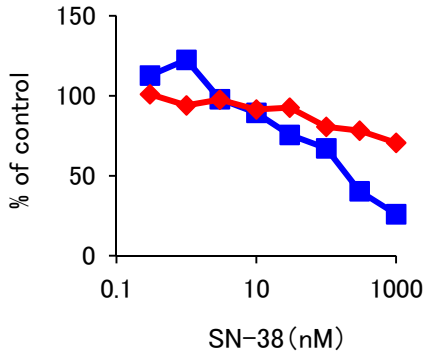
■ : Monolayer
◆ : Spheroid



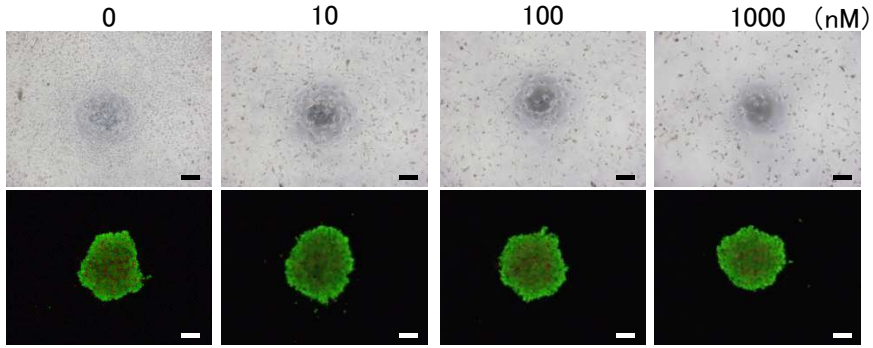
Scale bars : 200 μm
Upper : a phase-contrast microscope images
Lower : Live/Dead immunofluolor double staining images
Arrows (↙) : Cells in the peripheral regions are more susceptible to anticancer drugs than those in center region

Experiment I
Result Example 4: SN-38

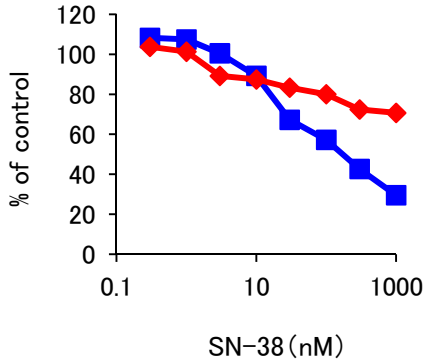
MDA-MB-231



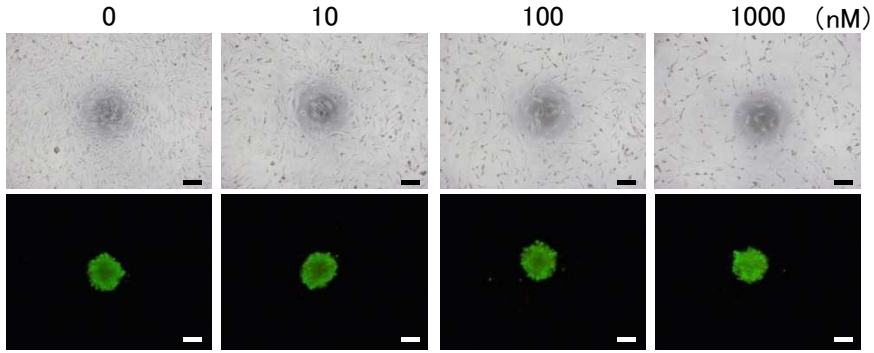
■ : Monolayer
◆ : Spheroid



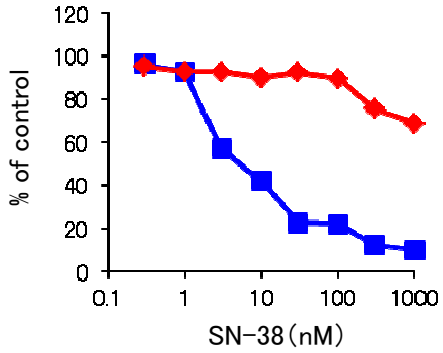
BT-549



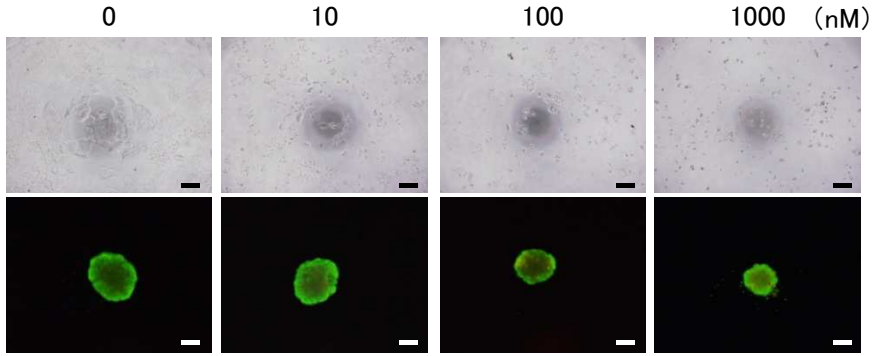
■ : Monolayer
◆ : Spheroid



MCF-7



■ : Monolayer
◆ : Spheroid



Scale bars : 200 μm
Upper : a phase-contrast microscope images
Lower : Live/Dead immunofluolor double staining images

Summary in Experiment I

Comparison of IC_{50} Value between Monolayer and Spheroid Culture

	CDDP（μM）		5-FU（μM）		DOC（nM）		SN-38（nM）	
Cells	Monolayer	Spheroid	Monolayer	Spheroid	Monolayer	Spheroid	Monolayer	Spheroid
MDA-MB-231	17.7	57.5	8.6	>300	3.9	>100	202.3	>1000
BT-549	46.7	205.2	5.7	>300	44.9	>100	172.3	>1000
MCF-7	13.8	98.7	2.2	>300	3.0	>100	5.3	>1000

IC_{50}: Monolayer Culture ＜ Spheroid Culture

Spheroid Cell Culture, using *PrimeSurface*, is exceptionally useful for Prediction of Anticancer Drug Penetration *in vivo* Solid Tumor

Experiment Ⅱ
Evaluation Examples of Anticancer Drug Efficacy
― Comparison of Drug Efficacy Mode ―

【 Culture Methods 】

Monolayer vs Spheroid (PrimeSurface)

【 Cells 】

HepG2 (Human Hepatocellular Liver Carcinoma Cell Line)

HeLa (Human Cervical Cervix Adenocarcinoma Cell Line)

【 Anticancer Drugs 】

5-FU(5-Fluorouracil) vs TPZ (Tirapazamine)

【 Evaluation Items 】

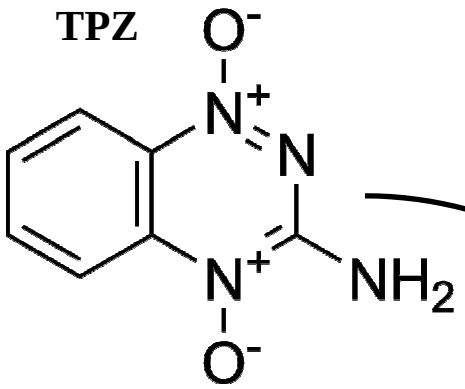
Viability assay by live cell ATP activity measurement

5-FU is used as a conventional cellular proliferation inhibitor

TPZ is a hypoxia triggered cytotoxic drug that causes DNA damages

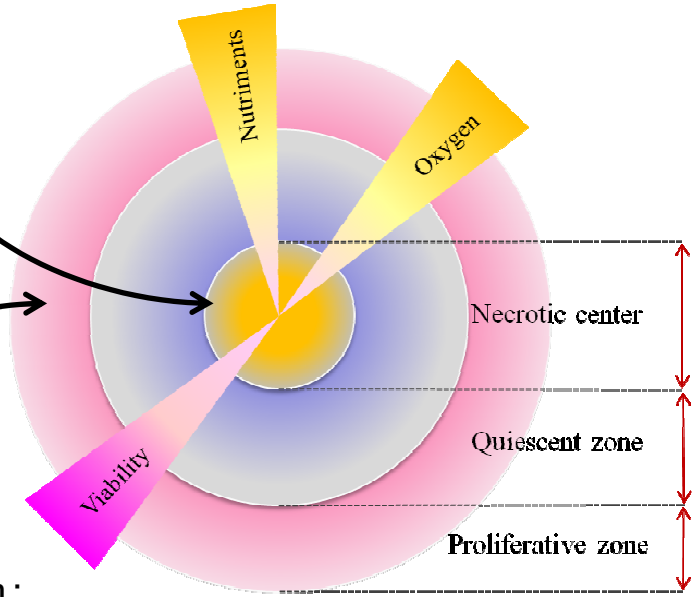
Experiment Ⅱ
The Difference of Drug Efficacy Mode between TPZ and 5-FU

TPZ

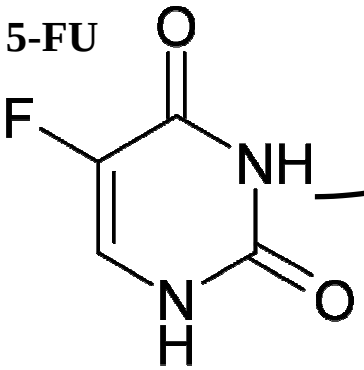


Mode of action:

- Effects hypoxic area with a low O_2 concentration
- Causes single & double strand breaks in DNA
- Reduces DNA synthesis
- **Damage in the hypoxic zone (especially in Spheroid structures)**



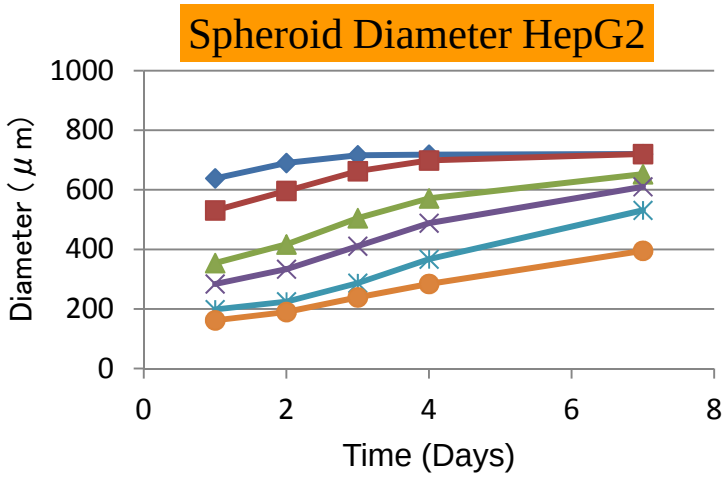
5-FU

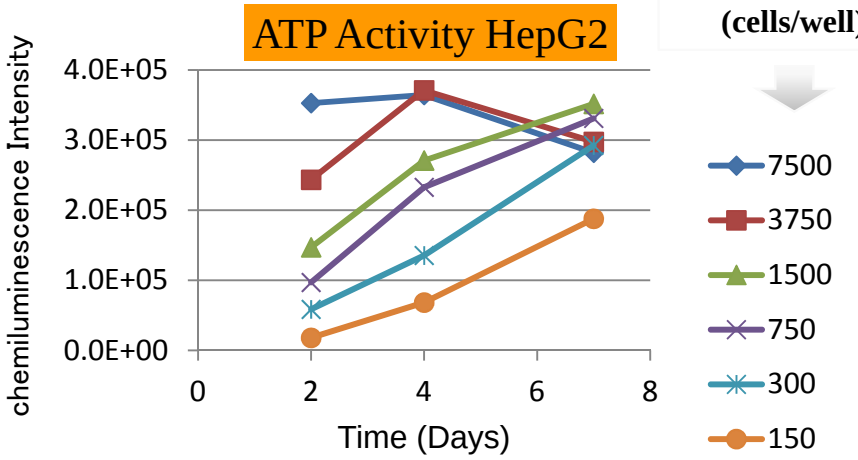


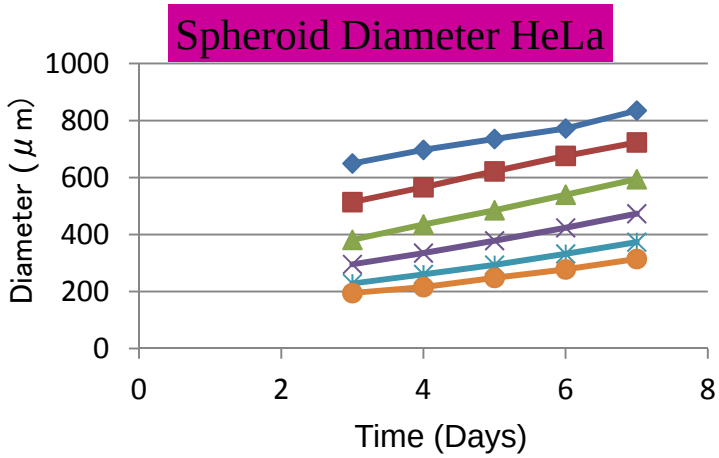
Mode of action:

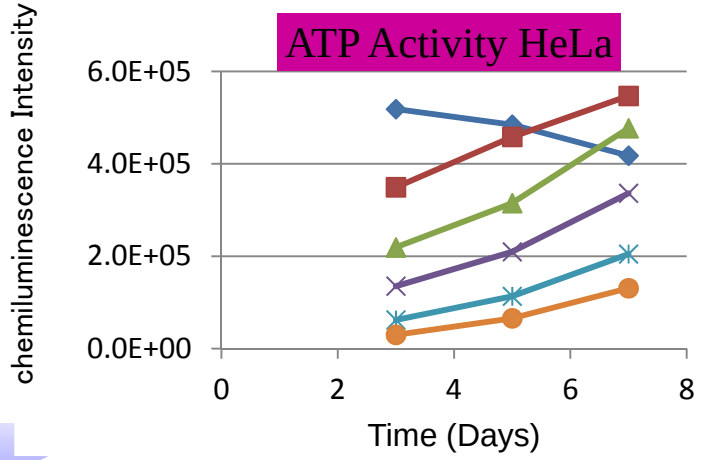
- Is effective against any cell type
- Effects the protein synthesis
- Inhibits the DNA replication
- **Damage in the proliferative zone (especially in Monolayer structures)**

Experiment Ⅱ
ATP Activity and Cell Proliferation Analysis for the Determination of Drug Efficacy Test Condition









The Optimal Cell Seeding Density was Determined to be 1,500 cells/well Because of Satisfactory Cell Proliferation and the Construction of Hypoxia Microenvironment

Experiment Ⅱ

Spheroid Formation and Size Change with Time in PrimeSurface 96 Multiwell Plate

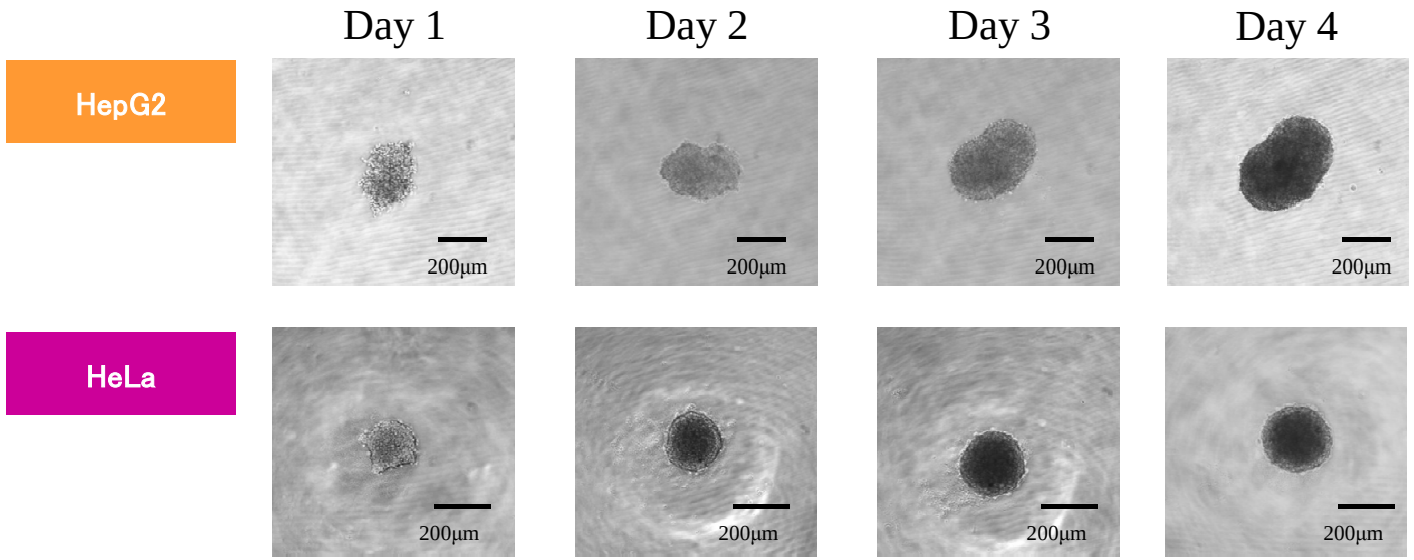
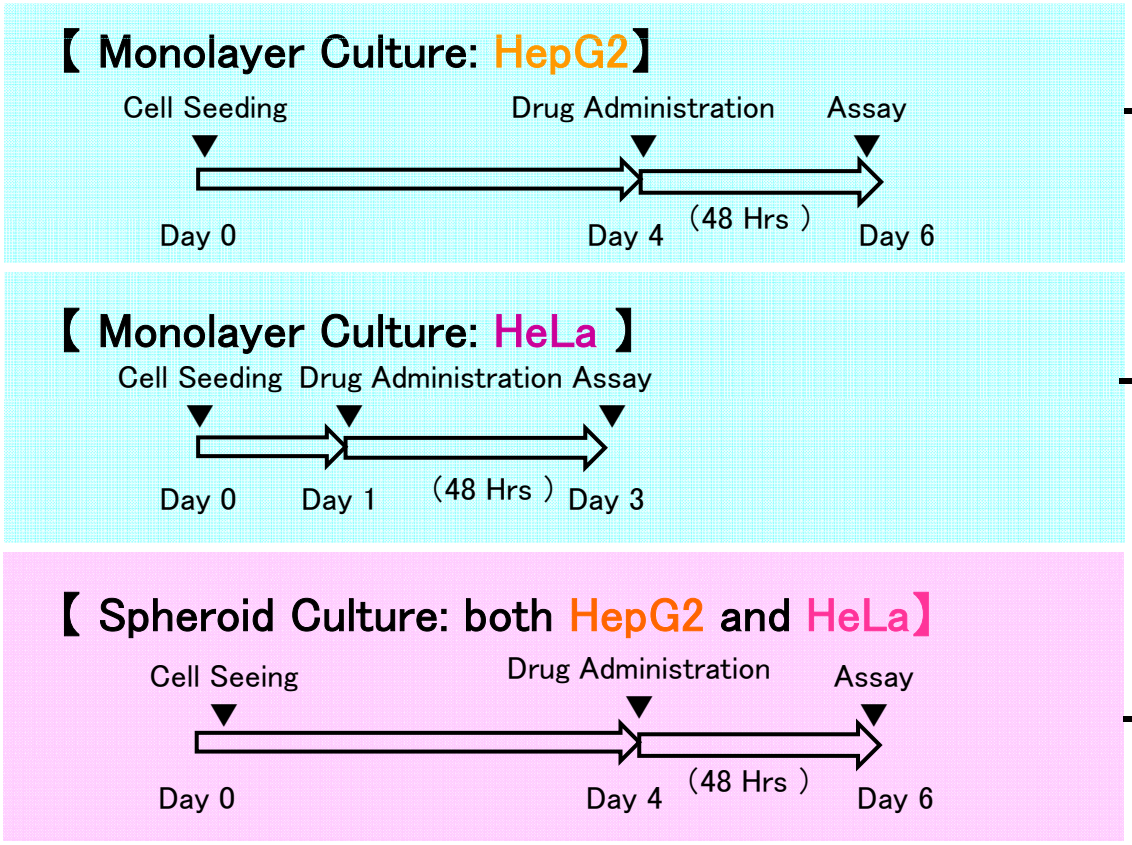


Plate	: PrimeSurface MS-9096U
Seeding Density	: 1,500 cells/well
Culture Medium	: HepG2･･･ DMEM Low Glucose + 10%FBS
	HeLa ･･･ MEM + 10%BS
Culture Period	: 4 Days

Satisfactory Cell Proliferation was Observed

Experiment Ⅱ
Cell Seeding , Drug Administrations and Assay Conditions



Drug administration periods are 48Hrs for both monolayer and spheroid culture in common.

【 Cell Seeding 】

Seed cells into wells into both the conventional into conventional 96 multiwell plate(MS-8096F) and PrimeSurfaceMS-9096U plate (1,500cells/100μL/well).

【 Drug Administration 】

① Aspirate and discard a 50 μL aliquot of culture medium at Day 4 (Monolayer Culture: HepG2), Day1 (Monolayer: HeLa) or Day 4 (Spheroid Culture: both HepG2 and HeLa).

② Add a 50μL aliquot of anticancer drug solution.

Experiment Ⅱ
Materials and Methods for Hypoxia observation with Lox-1 probe

【Prepare LOX-1 stock solution】

① Add 0.5 mL DMSO to LOX-1 tube and dissolve this by vortex mixer.
② Transfer the above solution into new 15 mlLtube.
③ Repeat ①-② steps three times. (To dissolve Lox-1 reagent completely and to prewash the tube)
④ Adjust the volume to 2.8mL by DMSO (1000 μ M LOX-1)
⑤ Sterilize1,000 μ M LOX-1 by filtration.
⑥ Transfer 0.5mL of 1,000 μ M LOX-1 to 1mL serum tube and store -20° C until use.

【Microscope observation of Hypoxia】

① Culture cells for 3 days
② Thaw 1,000 μ M LOX-1 stock solution.
③ Transfer 0.2 mL of 1,000 μ M LOX-1 stock solution into new 50mL tube and add 24.8 mL of culture medium.
(8 μ M LOX-1 in medium)
④ Add an 25 μ L aliquot of 8 μ M LOX-1 solution into each well of 96 well plate.
⑤ Observe the hypoxic region with microscope at the next day (= Day 4)

Experiment Ⅱ
Materials and Methods for Cell Viability Assay with CellTiter-Glo®

【Cell viability assay】

① Add the test compound to experimental wells, and incubate according to culture protocol.

② Equilibrate the plate and its contents at room temperature for approximately 30 minutes.

③ Add 100μL of CellTiter-Glo® Reagent (equal to the volume of cell culture medium present in each well) each plate (including standard curve plate). *If the top face of plate is wet, wipe the top face.

④ Shake the plate vigorously (*450 rpm*) for 15 minutes on an orbital shaker to induce cell lysis.

⑤ Allow the plate to incubate at room temperature for another 30 minutes (45 minutes total) to stabilize the luminescent signal.

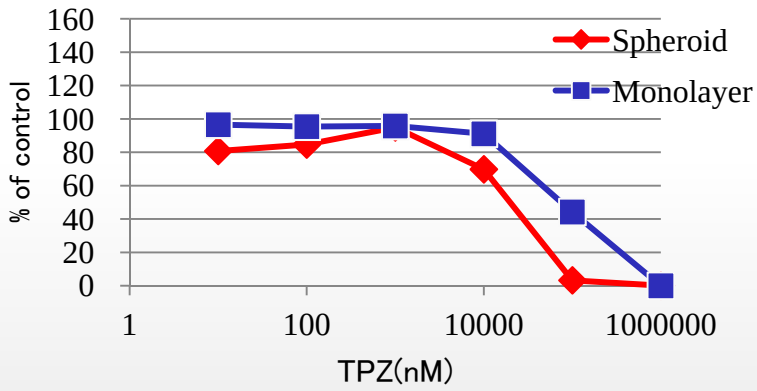
⑥ Transfer 100 μL of above ⑤ samples from each wells to the white-colored 96 well plate(MS-8096W).

⑦ Record luminescence. (Integration Time is 1.0sec) *Until measurement, protect samples from light.

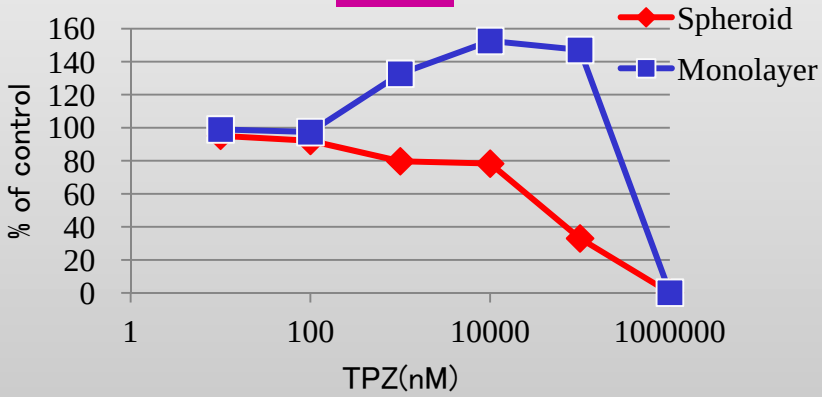
Experiment Ⅱ
TPZ & 5-FU Drug Efficacy on HepG2 & HeLa

TPZ

HepG2

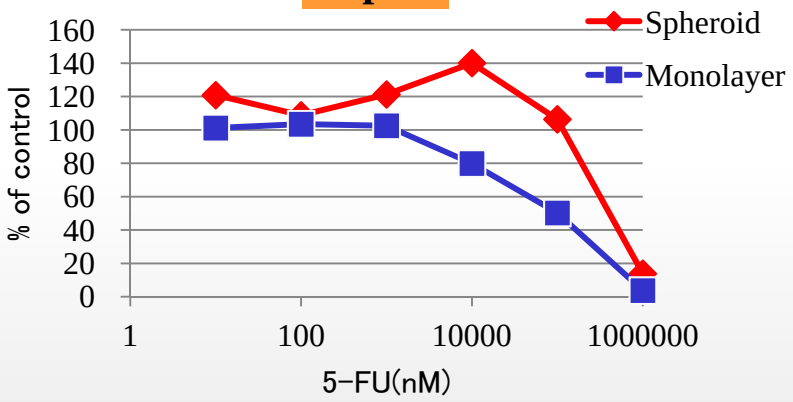


HeLa

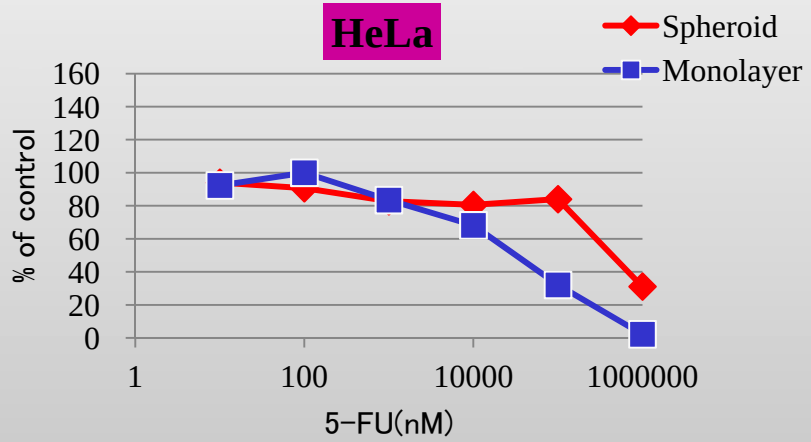


5-FU

HepG2



HeLa

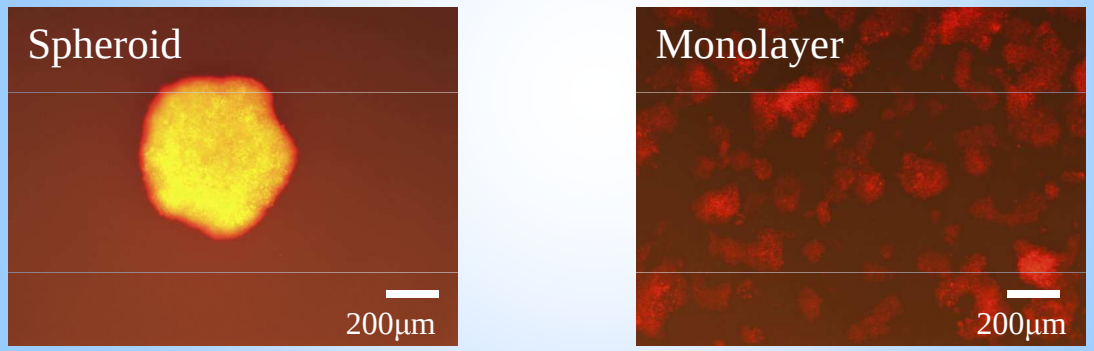


Summary in Experiment Ⅱ

Comparison of IC_{50} between TPZ and 5-FU

IC_{50}	TPZ(μM)		5-FU(μM)	
Cells	Monolayer	Spheroid	Monolayer	Spheroid
HepG2	75.3	19.8	101.1	406.7
HeLa	457.9	42.1	31.8	439.4

Hypoxia Observation in HepG2 Spheroid with Lox-1 Probe



- As Is Observed With Lox-1 Hypoxia Probe, Cells in Center Area of Spheroid are in Hypoxia Conditions.
- In Case Of TPZ, which is a Hypoxia Triggered DNA Damaging Cytotoxic Drug Showed Stronger Effect in *"Spheroid"* Than *"Monolayer"*
- These Two Observation Strongly Suggest 3D Drug Efficacy Test Environment Could be Reproduced with *"PrimeSurface"*.

Appendix

PrimeSurface is able to Produce *Uniform* Tumor Spheroid

In order to develop a robust assay and minimize variability, spheroids must be uniform in size and shape.

PrimeSurface, a culture ware designed for such screenings, provides the above solutions *!*

Z-Factor　Comparison between Monolayer and Spheroid Culture

HeLa	Monolayer	Spheroid
Day 3	0.86	0.70
Day 6	0.48	0.69

$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

- σ_p - SD of positive samples
- σ_n - SD of negative samples
- μ_p - mean of positive samples
- μ_n - mean of negative samples

Values for z-Factor:

1>Z>0.5 is a excellent assay, 0.5>Z>0 minimum acceptable assay and Z<0 unusable assay

See next page for experimental

【 Materials 】

- Used cells : HeLa Cells
- Medium : MEM with 10% BS
- PBS
- TrypLE Express
- Plate : PrimeSurface MS-9096W for Spheroid culture and MS-8096F for Monolayer culture

【 Methods 】

① Add 75 μ L of each cell suspension in a well of 96 well plate
(= 2,000 cells /1000 μ L×75 μ L/well = 1,500 cells/well) (See below Table)
② Incubate cells at 37° C in 5% CO_2 for 3days or 6days.

＜ Calculation of Z'-factor ＞

$$Z' = 1 - 3 \times (SD_{(cell+)} + SD_{(cell-)}) / (Mean_{(cell+)} - Mean_{(cell-)})$$

Table 1 Cell seeding condition

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 cells/well (Medium) 75μl	1,500 cell/well (add 75μl of 20,000 cells/ml)										0 cells/well (Medium) 75μl
B												
C												
D												
E												
F												
G												
H												

Other Information

- PrimeSurface can be stored at room temperature.
- The shelf life of PrimeSurface is two years after production.

"Sumitomo Bakelite Co. Ltd.", offers a variety of products based on its advanced plastic and polymer technology for the pharmaceutical researcher engaged in cell based assaying.

We will customize products at your request….

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