

Time-lapse imaging assay using the BioStation CT: A sensitive drug-screening method for three-dimensional cell culture

Ruriko Sakamoto,¹ M. Mamunur Rahman,² Manami Shimomura,³ Manabu Itoh⁴ and Tetsuya Nakatsura³

¹SCIVAX Corporation, Kawasaki, Kanagawa, Japan; ²SCIVAX USA Woburn, Massachusetts, USA; ³Division of Cancer Immunotherapy, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa, Chiba, Japan; ⁴SCIVAX Life Sciences, Kawasaki, Kanagawa, Japan

Key words

Breast cancer, drug sensitivity assay, imaging device, NanoCulture Plates, three-dimensional cell culture

Correspondence

Tetsuya Nakatsura, Division of Cancer Immunotherapy, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, 6-5-1 Kashiwanoha, Kashiwa 277-8577, Chiba, Japan.
Tel: 04-7131-5490; Fax: 04-7133-6606;
E-mail: tnakatsu@east.ncc.go.jp

Funding Information

This study was supported in part by a Third Term Comprehensive Control Research for Cancer grant from the Ministry of Health, Labor and Welfare, Japan.

Received February 16, 2015; Revised March 27, 2015;
Accepted March 31, 2015

Cancer Sci 106 (2015) 757–765

doi: 10.1111/cas.12667

Three-dimensional (3D) cell culture is beneficial for physiological studies of tumor cells, due to its potential to deliver a high quantity of cell culture information that is representative of the cancer microenvironment and predictive of drug responses *in vivo*. Currently, gel-associated or matrix-associated 3D cell culture is comprised of intricate procedures that often result in experimental complexity. Therefore, we developed an innovative anti-cancer drug sensitivity screening technique for 3D cell culture on NanoCulture Plates (NCP) by employing the imaging device BioStation CT. Here, we showed that the human breast cancer cell lines BT474 and T47D form multicellular spheroids on NCP plates and compared their sensitivity to the anti-cancer drugs trastuzumab and paclitaxel using the BioStation CT. The anticancer drugs reduced spheroid migration velocity and suppressed spheroid fusion. In addition, primary cells derived from the human breast cancer tissues B58 and B61 grown on NCP plates also exhibited similar drug sensitivity. These results were in good agreement with the conventional assay method using ATP quantification. We confirmed the antitumor effects of the drugs on cells seeded in 96-well plates using the BioStation CT imaging technique. We expect this method to be useful in research for new antitumor agents and for drug sensitivity tests in individually-tailored cancer treatments.

The investigation of human cancers is largely reliant on the use of established cancer cell lines *in vitro*. Cancer cell lines are generally cultured on flat glass or plastic plates, wherein these cells grow in a two-dimensional (2D) fashion and form monolayer sheets. Monolayer cell cultures insufficiently reflect the physiological response pattern of the *in vivo* situation due to the fundamental geometric differences between 2D cultures and three-dimensional (3D) solid tumors. When cancer cells are cultured in 2D, they lose their polarity; moreover, the locations of membrane receptors on the cell surface differ from those *in vivo*, because tissues and organs are 3D in nature.^(1–3) A 3D architecture for tumor cells needs to be generated *in vitro* to simulate the multicellular microenvironment when investigating tumor cell physiology and the response to therapeutic agents.⁽⁴⁾

To eliminate the drawbacks of 2D culture, several methods have recently been developed for 3D cell culture.^(5,6) Multicellular tumor spheroids have been widely used as an *in vitro* 3D model to evaluate tumor cell proliferation, apoptosis, differentiation, gene expression and metabolism in a multicellular context.^(7,8) Tumor cells in spheroids show higher degrees of morphological^(9,10) and functional^(11,12) differentiation than those of cells grown in monolayer culture. They also differ in their responses to cell signaling-related reagents compared with 2D cultured cell lines.^(13–16) Moreover, definitive differences

have been shown with regard to apoptotic sensitivity to chemotherapeutic agents in nonmalignant and malignant mammary cell lines between 2D and 3D cultured cells.⁽¹⁷⁾

To form multicellular clusters, attachment between cells and the tissue culture plate needs to be reduced. This requires either the addition of certain supplements to the culture medium, such as collagen gel, or surface treatment of the tissue culture plate. However, these methods are usually complex and difficult to perform. The NanoCulture Plate (NCP) is a new system devised for 3D culture.^(18,19) The NCP has a fine pattern on the well bottom surface, which provides scaffolding for cells with expanding pseudopodia. Thus, the cells can move and aggregate to form spheroids on the NCP. These spheroids further migrate and fuse to each other during culture.⁽¹⁸⁾

Cell motility, migration and adhesion are key factors in cancer cell biology.^(20–23) Therefore, it is vital to develop a new method to quantify cell migration in a 3D system to help in the screening of anti-cancer drugs. In this study, we analyze the sensitivity of human breast cancer cell lines and primary cells derived from human breast cancer tissue to anti-cancer drugs using NCP-cultured cells. Furthermore, we propose a novel drug assay method using NCP together with an imaging analysis system. We demonstrate the reproducibility of this imaging method and also compare its cytotoxicity

quantification with that using the conventional luminescent ATP assay.

Materials and Methods

Cell line culture and seeding. The human breast cancer cell lines BT474 and T47D were maintained on plastic tissue culture plates in RPMI1640 medium (Sigma ALDRICH, St. Louis, MO, USA) with 10% heat-inactivated FBS. The cells were dissociated from the plastic tissue culture plates (TCP) using Accumax (Innovate Cell Technologies, San Diego, CA, USA), seeded at a density of 1×10^4 cells per well in a 96-well NCP (SCIVAX Life Sciences, Kanagawa, Japan) or TCP and cultured in M medium (SCIVAX Life Sciences).

Addition of drugs. The working solutions of trastuzumab or paclitaxel were diluted with M medium to two times concentration for assay. On day 3 after cell seeding, we removed 50% of the medium from the well and added the working solutions of drugs in a volume equal to that removed. The cells were then cultured until further use.

Time-lapse phenotypic assay. Time-lapse images were obtained using a BioStation CT (Nikon, Tokyo, Japan) incubator equipped with a camera for video imaging. For these experiments, cell/spheroid migration was filmed for 7 days after drug introduction. We analyzed the images using the image analysis software CL-Quant Ver. 2.0 (Nikon).^(24,25)

Results

The breast cancer cell line BT474 formed uniform spheroids on NanoCulture Plates. Seeding of cells on NCP was performed using the same method for 2D culture on TCP. BT474 is a breast cancer cell line that expresses HER2.⁽²⁶⁾ When BT474 cells were suspended in medium and seeded on NCP and TCP at the same density, they formed several uniform spheroids after 3 days in NCP wells and formed a monolayer in TCP wells. These spheroids are semispherical in shape with a diameter of approximately 100 μm (Fig. 1a). Moreover, the growth curve of BT474 cells seeded on NCP resembled that on TCP (Fig. 1b). The spheroids migrated continuously and fused on the NCP, forming larger spheroids, thus reducing the spheroid number by day 7 (Fig. 1c).

Drug assay for BT474 cell spheroids on NanoCulture Plates. We added the anti-cancer drug trastuzumab or paclitaxel in various concentrations to the BT474 spheroids on NCP. Based on microscopic observations, we noted that the growth of these spheroids was suppressed after 7 days compared with spheroid growth in non-treated wells (Fig. 2a,b). We observed many damaged cells scattered around the spheroids in the drug-treated wells. Spheroid size was sensitive to drug concentration, and 1 $\mu\text{g}/\text{mL}$ trastuzumab-treated spheroids were smaller than non-treated spheroids, while the 10 $\mu\text{g}/\text{mL}$ trastuzumab-treated spheroids were even smaller (Fig. 2a). Dose-dependent effects of paclitaxel on spheroid size were also observed (Fig. 2b).

We quantified ATP in the samples treated with trastuzumab or paclitaxel. These drugs appeared to act effectively against the BT474 spheroids in a dose-dependent manner (Fig. 2c); however, the effect of 0.1 $\mu\text{g}/\text{mL}$ trastuzumab on BT474 spheroids was negligible. The ATP concentration was decreased to 78% with the 1 $\mu\text{g}/\text{mL}$ treatment and to 62% with the 10 $\mu\text{g}/\text{mL}$ treatment, compared with the ATP concentration in the drug-free condition. For paclitaxel, the ATP concentration was decreased to 32% with the 0.06 $\mu\text{g}/\text{mL}$ treatment, to 8% with the 0.6 $\mu\text{g}/\text{mL}$ treatment and to 6%

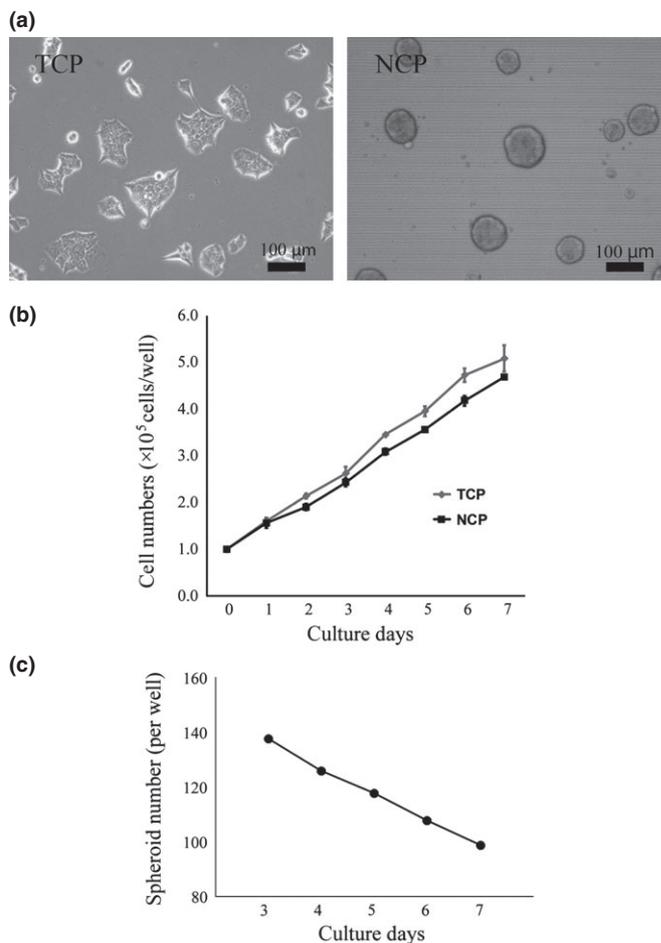


Fig. 1. The breast cancer cell line BT474 cultured in two-dimensional (2D) and three-dimensional (3D) conditions. (a) Phase-contrast images of BT474 cells cultured for 3 days on plastic tissue culture plates (TCP) and on NanoCulture Plates (NCP). Scale bar: 100 μm . (b) The growth curves of BT474 cells seeded on TCP and NCP. Bars, \pm SD ($n = 3$). (c) The spheroid fusion effect is reflected by spheroid number/well over time.

with the 6 $\mu\text{g}/\text{mL}$ treatment, compared with the ATP concentration in the drug-free condition. We showed that the spheroids cultured in NCP could be similarly assayed using an identical ATP quantification method. However, this method requires cell solubilization, and the sample cells are killed and, thus, cannot be used for another assay. Furthermore, changes in the survival rate could not be observed using this method.

Drug assays using BT474 and T47D spheroids on NanoCulture Plates combined with an imaging analyzer. Migration and fusion of cells/spheroids were observed on NCP. Spheroid size increased mainly through migration and fusion on NCP (Figs 3a,b [upper panel] and Suppl. Video S1a). In the wells treated with high drug concentrations, spheroid size was smaller than that in the drug-free well (Fig. 2a,b). We believe that spheroid migration and fusion on NCP are suppressed by the effects of the drug, and that the effect of the drug can be estimated by measuring the number and migration velocity of spheroids in time-series analyses.

We demonstrated the effects of trastuzumab against BT474 spheroids (Fig. 3a,c,d and Suppl. Video 1Sb–d). BT474 spheroids on NCP were observed in the presence and absence of trastuzumab using the BioStation CT. Dose-dependent

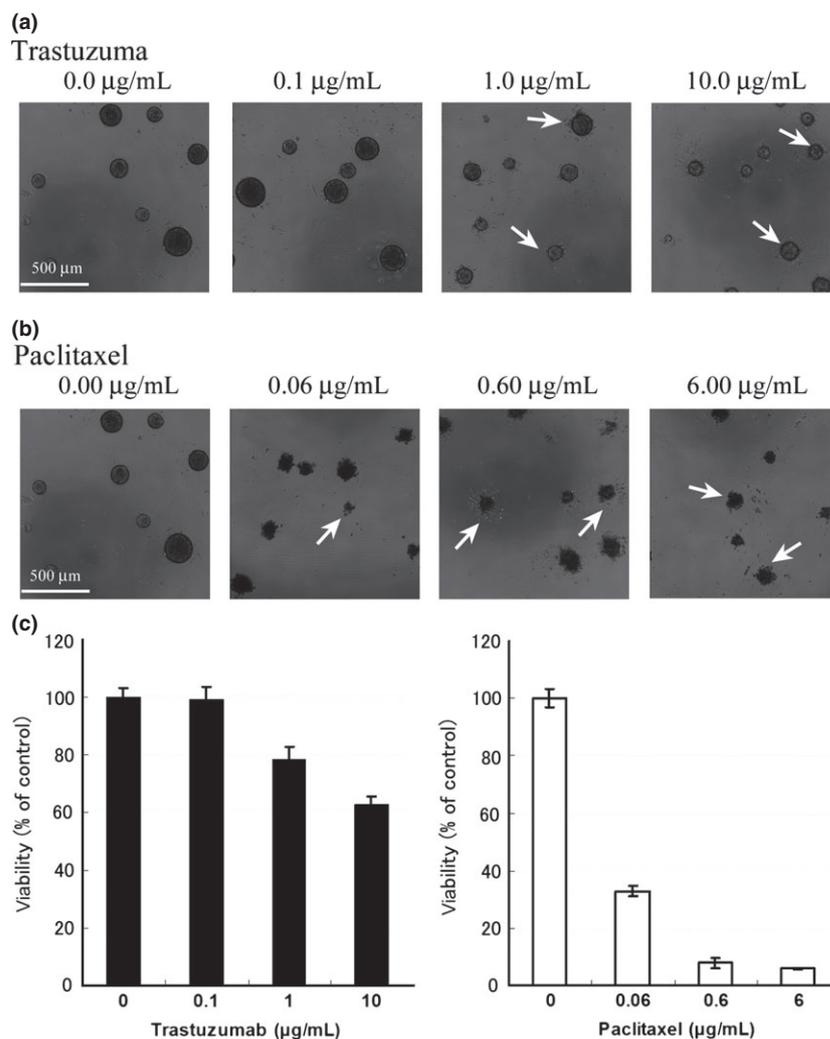


Fig. 2. Drug assay in BT474 spheroids grown on Nanoculture Plates (NCP). BT474 cells were seeded on NCP. After 3 days, drugs were added to the wells and cultured to day 7. (a) Phase-contrast images of BT474 cell spheroids taken on day 7 after treatment with trastuzumab (0, 0.1, 1 and 10 µg/mL). The white arrow shows damaged cells. Scale bar: 0.50 mm. (b) Phase-contrast images of BT474 cell spheroids taken on day 7 after treatment with paclitaxel (0, 0.06, 0.6 and 6 µg/mL). The white arrow shows the damaged cells. Scale bar: 0.50 mm. (c) Graph showing the viability of cells at the endpoint. The number of viable cells was estimated by ATP quantification. Bars, \pm SD ($n = 3$).

decreases in the migration velocity and spheroid size were noted (Fig. 3a and Suppl. Video S1b–d). In wells with or without 0.1 µg/mL trastuzumab, the spheroids migrated and fused with each other. In wells with 1 or 10 µg/mL trastuzumab, spheroid migration and fusion were suppressed, and spheroids were smaller than those in untreated wells. The average velocity magnitude of all spheroids in images taken at 1-h intervals during the observation period are shown in Figure 3(c). The spheroids moved at an average of 5.37 pixels per hour during the observation period in the drug-free well (red line); however, the average movement in the trastuzumab-containing well (blue line) decreased in a dose-dependent manner, with a decrease in velocity clearly observed later during the observation period. These results suggest that spheroid migration was inhibited by trastuzumab at high concentrations. The spheroid numbers over the time course are shown in Figure 3(d). The number of total spheroids in the images gradually decreased throughout the observation period in the drug-free well. In the trastuzumab-treated well, the decrease in spheroid numbers was suppressed by the 1 and 10 µg/mL concentrations (Fig. 3d), suggesting obstruction of spheroid fusion as a result of the trastuzumab-mediated inhibition of spheroid migration, as shown in Figure 3(c). These results correlate with the decrease in viability, as noted by ATP quantification (Fig. 2c).

Next, we demonstrated the effects of paclitaxel on BT474 spheroids (Fig. 3b–d and Suppl. Video S1e–g). An obvious

decrease in spheroid migration velocity was seen after the addition of paclitaxel (Fig. 3b and Suppl. Video S1e–g), and less migration and fusion of spheroids were seen in the well containing paclitaxel. The average velocity magnitude per hour of the spheroids in the paclitaxel-containing well during the observation period (blue line) was significantly reduced compared with that in the drug-free well (red line) (Fig. 3c). The reduction in total spheroid number by fusion was also clearly suppressed by paclitaxel (Fig. 3d). Spheroid numbers were decreased to 55% in the drug-free well, to 75% in the 0.06 µg/mL paclitaxel-treated well and to 85% in the 0.6 and 6 µg/mL paclitaxel-treated wells after 7 days. Contrary to expectations, 0.06 µg/mL paclitaxel was effective, and the effect of 6 µg/mL paclitaxel was no greater than that of 0.6 µg/mL, suggesting little difference between the 0.6 and 6 µg/mL paclitaxel concentrations.

To confirm the utility of these methods, we performed a drug assay using another breast cancer cell line, T47D, and the BioStation CT (Fig. 4c,d and Suppl. Video S2). T47D expresses low levels of HER2.⁽²⁶⁾ T47D seeded on NCP formed spheroids resembling those of BT474 cells (Fig. 4a). T47D spheroids showed a high sensitivity to paclitaxel, but, according to microscopic observation, did not respond to trastuzumab (Fig. 4a). Moreover, ATP quantification in T47D spheroids also showed high sensitivity to paclitaxel and a low response to trastuzumab (Fig. 4b), confirming low HER2

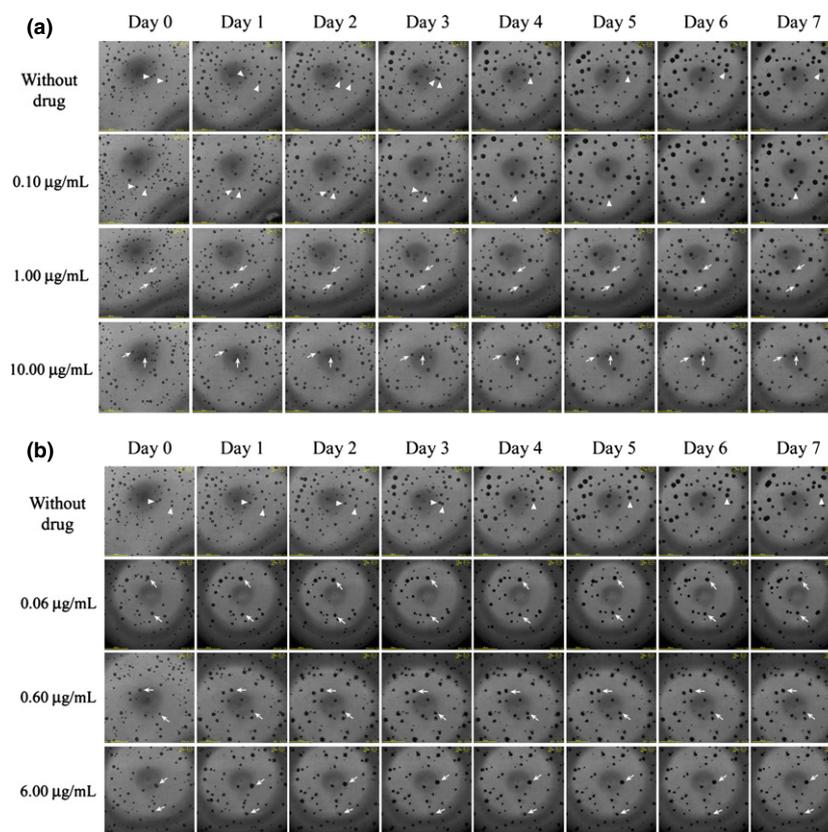


Fig. 3. Time-lapse imaging of drug sensitivity of BT474 spheroids on Nanoculture Plates (NCP). BT474 cells were seeded on NCP, drugs were added and time-lapse imaging was initiated on day 3. Plate images were obtained at 1-h intervals for 7 days. (a) Time-lapse images of BT474 cells cultured with or without trastuzumab are shown. The white arrow indicates selected spheroids in the well. Scale bar: 1.00 mm. (b) Time-lapse images of BT474 cells cultured with or without paclitaxel are shown. The white arrow indicates selected spheroids in the well. Scale bar: 1.00 mm. (c) The average velocity magnitude of all spheroids in the images at 1-h intervals. Red line: average velocity magnitude per hour in the drug-free well during the observation period. Blue line: average velocity magnitude per hour at various concentrations in the drug-containing wells during the observation period. (d) The number of spheroids in each well with or without the drug over the entire time course.

expression in T47D spheroids. In the BioStation CT assay, the spheroids moved at an average of 7.19 pixels per hour during the observation period in the drug-free well (red line); however, a 68% reduction in spheroid migration velocity was seen with the addition of paclitaxel (Fig. 4c and Suppl. Video S2c), but only a 6% reduction with trastuzumab (Fig. 4c and Suppl. Video S2b). The decrease in total spheroid number was clearly suppressed by paclitaxel, but not by trastuzumab (Fig. 4d). These results show that the effect of anti-cancer drugs against cancer cell lines on NCP could be evaluated effectively using the BioStation CT.

Drug assays using primary cells derived from breast cancer tissues on NanoCulture Plates combined with an imaging analyzer. It was confirmed that several primary tumor cells derived from the liver, lung, pancreas (data not shown) and breast cancer tissues can form spheroids on NCP (Fig. 5a). Drug assays in such primary cells can be performed using NCP and the BioStation CT. The primary cells B58 and B61 derived from human breast cancer tissues contain large amounts of debris; however, they successfully formed heterogeneous spheroids (Fig. 5a). Cell immunostaining confirmed that these spheroids express the epithelial cell marker cytokeratin 7/17 (Fig. 5b). Large amounts of debris in the well interfere with imaging analysis using the BioStation CT. Therefore, we digested the spheroids using collagenase to obtain single cells and passaged them in the new NCP wells to eliminate debris. After the cells reformed spheroids, we added paclitaxel

to the wells and initiated the drug assay using the BioStation CT for two samples derived from the human breast cancer tissues B58 and B61 (Fig. 5c,d and Suppl. Video S3). It was difficult to evaluate the suppression of the drug-induced decrease in total spheroid number, because the spheroids did not show complete fusion as observed in the cancer cell lines; however, spheroid migration could be observed (Fig. 5c, white arrow). Spheroid migration was suppressed by paclitaxel (Fig. 5c). The average velocity magnitude of the spheroids in images taken at 1-h intervals during the observation period are shown in Figure 5(d). The average velocity magnitude per hour of the spheroids in paclitaxel-treated wells (blue line) was clearly decreased compared with that in the drug-free well (red line) for each sample. The suppression of migration by paclitaxel was more suppressed in B58 than B61 spheroids (Fig. 5d). The quantification of ATP in each sample is shown in Figure 5(e). These results correlate with the decrease in spheroid migration velocity (Fig. 5d).

On the basis of our results, we consider that the effect of anticancer drugs against primary cells derived from breast cancer tissue could be successfully evaluated using the techniques described here.

Discussion

It is reported that malignant or nonmalignant mammary epithelial cells, as well as other differentiated cell types, lose many

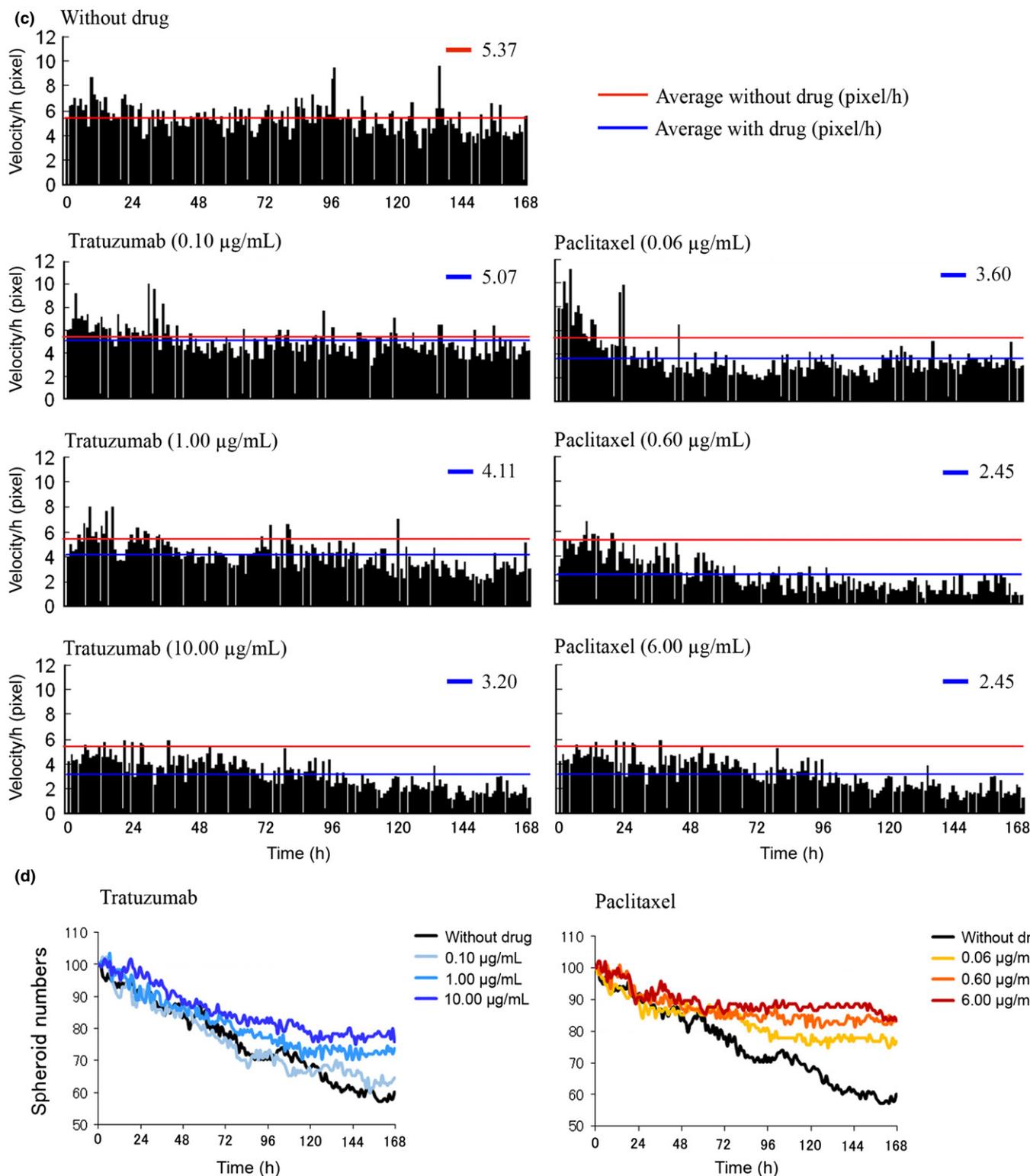


Fig. 3. Continued.

aspects of differentiation upon dissociation and culture in 2D on TCP.^(27–30) Recently, 3D tumor models have emerged as valuable *in vitro* research tools, although the power of such systems as quantitative reporters of tumor growth and treatment response has not been adequately explored.

The conventional methods used for 3D cell culture are complicated. In contrast, the same procedures for seeding and cul-

turing of cells on TCP can be performed as those on TCP commonly used for 2D cell culture. In 3D cell culture methods using a collagen gel or agar, spheroids grow from single cells for approximately 2–3 weeks.^(22,31,32) However, cell migration and spheroid formation on NCP takes only approximately 3–5 days.⁽³³⁾ It is easy to compare the results obtained from NCP with those from 2D cell cultures. Moreover, sampling errors in

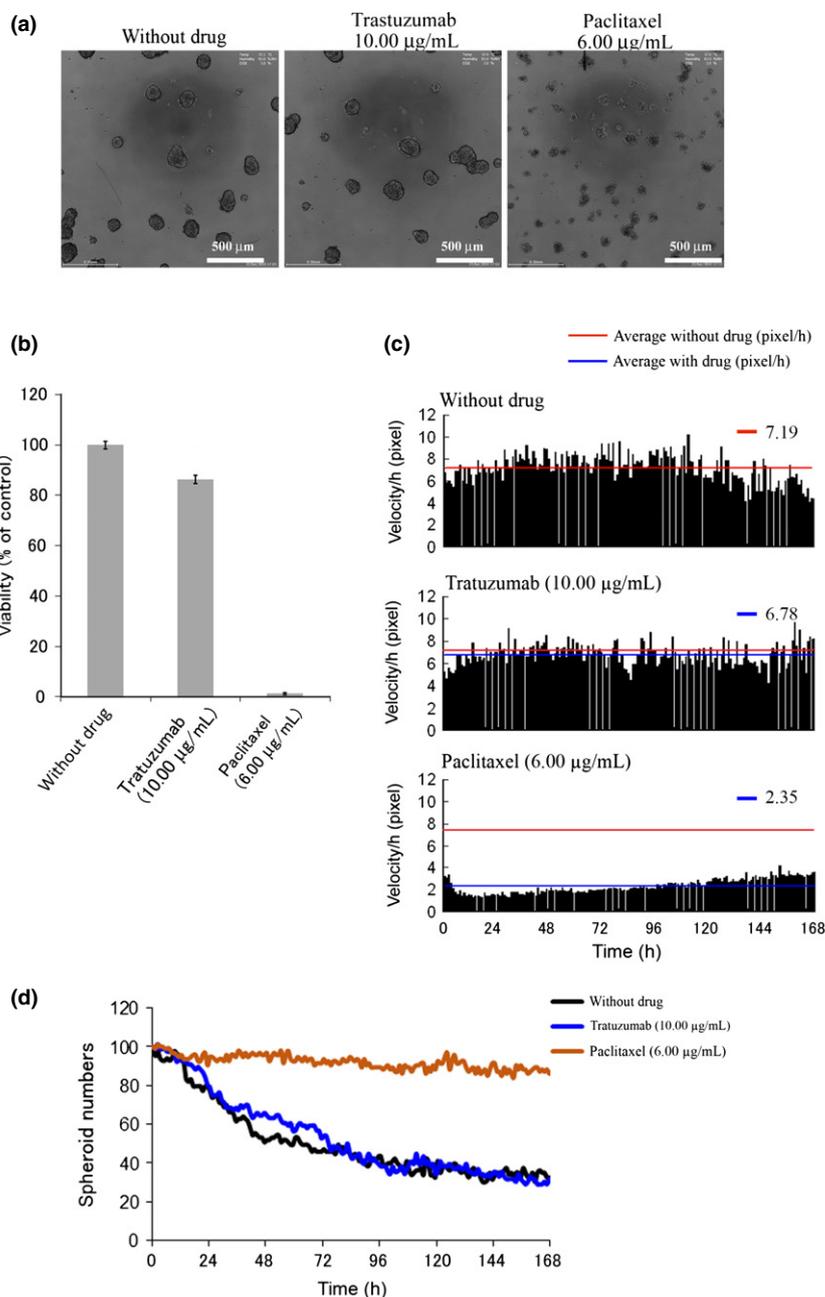


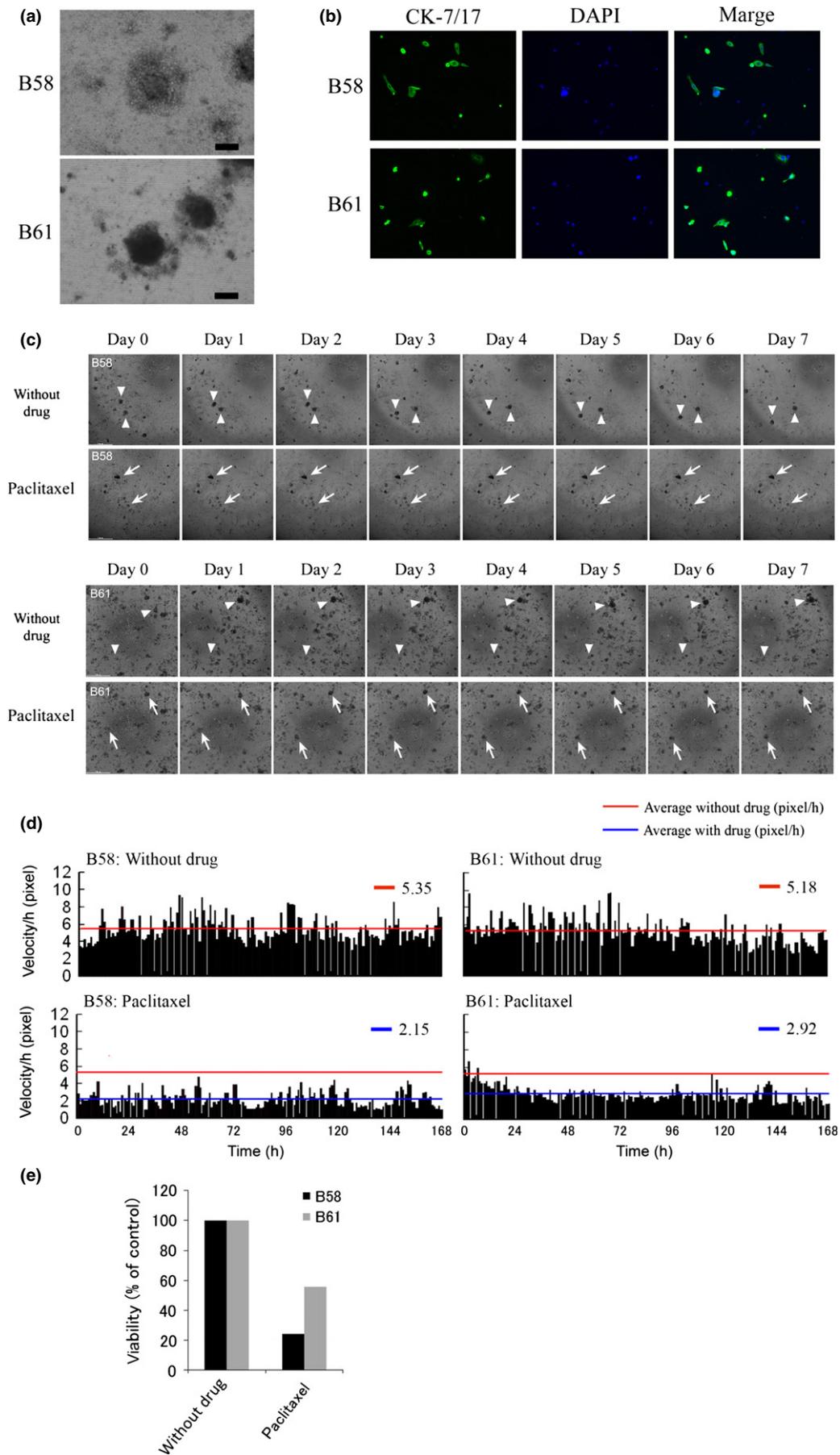
Fig. 4. Drug assay in T47D spheroids on Nanoculture Plates (NCP). T47D cells were seeded on NCP, and on day 3, drugs were added and time-lapse imaging was initiated. Plate images were obtained at 1-h intervals for 7 days. (a) Phase-contrast images of T47D cells cultured with 10 µg/mL trastuzumab or 6 µg/mL paclitaxel or no drugs on day 7. Scale bar: 0.50 mm. (b) The graph shows the viability of the cells at the endpoint. The number of viable cells was estimated using ATP quantification. Bars, \pm SD ($n = 3$). (c) The average velocity magnitude of all spheroids in the images at 1-h intervals. Red line: average velocity magnitude per hour in the drug-free well during the observation period. Blue line: average velocity magnitude per hour in the drug-containing wells during the observation period. (d) The number of spheroids in each well with or without drugs over the entire time course.

NCP-cultured cells are not significant between experiments, because the spheroids formed in NCP are better in shape and size and can be easily observed microscopically. It is possible to measure the location, form and size of spheroids from the obtained images.

We demonstrated the effectiveness of an imaging-based approach in the analysis of tumor growth and drug treatment

response using an *in vitro* 3D model of breast carcinoma. In this study, we used NCP and the BioStation CT to demonstrate a novel approach for drug assays in cancer cells. Using this system, we introduced a model of the growth mechanism for adherent cancer cells on NCP based on migration and fusion behavior. The BioStation CT is a time-lapse imaging analyzer. When the TCP are placed in the BioStation CT, plate images

Fig. 5. Time-lapse imaging for drug sensitivity of primary breast cancer cells. Primary cells derived from human breast cancer tissues (sample ID: B58 and B61) were seeded on Nanoculture Plates (NCP). After 3 days, spheroids were collected, digested into single cells and passaged into fresh NCP wells. The cells were cultured for 3 days and then treated with the drugs (6 µg/mL paclitaxel), and time-lapse imaging was initiated. Plate images were obtained at 1-h intervals for 7 days. (a) Phase-contrast images of primary cell cultures on day 3. Scale bar: 100 µm. (b) Immunofluorescence image of cells derived from the same tissues used for time-lapse imaging. Cells were cultured on NCP for 10 days, digested using collagenase and seeded onto a slide chamber. The cells were stained for cytokeratin 7/17 (an epithelial cell marker, green) and with 4',6-diamidino-2-phenylindole (blue) for nuclear staining. (c) Time-lapse image of primary cells (passage 1) cultured with 6 µg/mL paclitaxel or no drugs. Scale bar: 0.50 mm. The white arrow indicates selected spheroids in the well. (d) The average velocity magnitude of all spheroids in the images at 1-h intervals. Red line: average velocity magnitude per hour in the drug-free well during the observation period. Blue line: average velocity magnitude per hour in the drug-containing wells during the observation period. (e) The graph shows the viability of the cells at the endpoint. The number of viable cells was estimated by ATP quantification.



can be obtained at a fixed position and at constant intervals. The migration velocity and the number of spheroids derived from the cancer cell lines grown on NCP can, thus, be measured using the BioStation CT. The change in survival rate during the observation period can also be observed using this technique. Furthermore, we can calculate the total spheroid area, and this system can be used to evaluate both cell motility and tumor growth. We cannot perfectly distinguish live from dying spheroids, so there is a limitation in the evaluation of tumor growth through only calculating the total spheroid area. This method is superior to ATP and other proliferation assays for drug screening, because sample cells are killed in those assays and cannot be used for subsequent assays. In addition, the change in survival rate cannot be observed by those methods.

Migration is an important feature of cancer cell drug screening.^(21,23) Compared with other common 3D plates (i.e. ultra low attachment plates, U-bottom plates or the hanging drop system), cells on NCP can migrate and form spheroids rather than aggregate. Drugs, such as docetaxel, are not effective on prostate carcinoma cell (DU145) spheroids in U-bottom plates but are effective on NCP (R. Sakamoto, M Ito, unpublished data). It was shown that spheroid morphology is a vital area for chemotherapeutic drug screening.^(34,35) It is not sufficient to take an image stack and analyze it at a single time point.⁽³¹⁾ The imperative feature of the time-lapse phenotypic assay is to analyze cell movement after drug treatment. When we cultured both HER2-positive (BT474) and HER2-negative (T47D) cells on NCP, BT474 spheroid velocity was lower than that of T47D (5.37 vs 7.19) in drug-free wells. Complex chromosome alterations affecting chromosomes 8, 11 and 17 are frequently observed in HER2-positive cell lines. These chromosomes contain genes that are commonly involved in cell migration, invasion and metastasis. Thus, HER2-positive cell lines show

structural aberrations in comparison with HER2-negative cells.^(13,36) The Biostation CT allows for the display of minimum sensitivity of cell spheroids in a 3D system.

We believe that drug assays of primary tumor cells derived from human cancer tissue can be performed using this technique. We have confirmed that several primary tumor cells derived from the liver, lung, pancreas and breast cancer tissue can form spheroids on NCP (M. Shimomura, R. Sakamoto, unpublished data). It is possible to estimate the effects of drugs on cells seeded in a single well in a 96-well plate using this technique in combination with the BioStation CT, and we expect that it will be helpful in determining the effect of chemotherapeutic agents on human cancer cells from primary clinical specimens that are often available in very small amounts.

In this paper, we propose an innovative method for conducting drug assays. The effect of antitumor agents can be estimated by measuring the migration velocity of spheroids on NCP in a time-series analysis. We expect this method will be useful in research on new antitumor agents and in drug sensitivity tests for individually tailored cancer treatments.

Acknowledgments

We thank Noboru Hirakawa and Hirotada Watanabe (Nikon Corporation) for the analysis of the imaging data from the BioStation CT. We also thank Noriko Sakemura, Noriaki Wada, Satoshi Fujii and Chisako Yamauchi for providing the tumor samples.

Disclosure Statement

Tetsuya Nakatsura is supported by a funding from SCIVAX co, Ltd. The other authors have no potential conflicts of interest to declare with regard to this study.

The authors have no conflict of interest to declare.

References

- Baker BM, Chen CS. Deconstructing the third dimension-how 3D culture microenvironments alter cellular cues. *J Cell Sci* 2012; **125**: 1–10.
- McCaffrey LM, Macara IG. Epithelial organization, cell polarity and tumorigenesis. *Trends Cell Biol* 2011; **21**: 727–35.
- Raghavan S, Shen CJ, Desai RA, Sniadecki NJ, Nelson CM, Chen CS. Decoupling diffusional from dimensional control of signaling in 3D culture reveals a role for myosin in tubulogenesis. *J Cell Sci* 2010; **123**: 2877–83.
- Saar M, Zhao H, Nolley R *et al*. Spheroid culture of LuCaP 147 as an authentic preclinical model of prostate cancer subtype with SPOP mutation and hypermutator phenotype. *Cancer Lett* 2014; **351**: 272–80.
- Lee GY, Kenny PA, Lee EH, Bissell MJ. 3D culture models of normal and malignant breast epithelial cells. *Nat Methods* 2007; **4**: 359–65.
- Haycock JW. 3D cell culture: a review of current approaches and techniques. *Methods Mol Biol* 2011; **695**: 1–15.
- Mueller-Klieser W. Multicellular spheroids: a review on cellular aggregates in cancer research. *J Cancer Res Clin Oncol* 1987; **113**: 101–22.
- Mueller-Klieser W. Tumor biology and experimental therapeutics. *Crit Rev Oncol Hematol* 2000; **36**: 123–39.
- Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. *Cell* 2007; **130**: 601–10.
- Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science* 1988; **240**: 177–84.
- Nyga A, Cheema U, Loizidou M. 3D tumor models: novel *in vitro* approaches to cancer studies. *J Cell Commun Signal* 2011; **5**: 239–48.
- Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* 2007; **8**: 839–45.
- Pickl M, Ries CH. Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. *Oncogene* 2009; **28**: 461–8.
- Wang F, Weaver VM, Petersen OW, Larabell CA, Dedhar S, Briand P. Reciprocal interactions between beta-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. *Proc Natl Acad Sci USA* 1998; **95**: 14821–6.
- Liu H, Radisky DC, Wang F, Bissell MJ. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. *J Cell Biol* 2004; **164**: 603–12.
- Weigelt B, Lo AT, Park CC *et al*. HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment. *Breast Cancer Res Treat* 2010; **122**: 35–43.
- Weaver VM, Lelievre S, Lakin JN, Chrenek MA, Jones JC, Giancotti F. Beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. *Cancer Cell* 2002; **2**: 205–6.
- Yoshii Y, Waki A, Yoshida K *et al*. The use of nanoimprinted scaffolds as 3D culture models to facilitate spontaneous tumor cell migration and well-regulated spheroid formation. *Biomaterials* 2011; **32**: 6052–8.
- Karlsson H, Fryknäs M, Larsson R, Nygren P. Loss of cancer drug activity in colon cancer HCT-116 cells during spheroid formation in a new 3-D spheroid cell culture system. *Exp Cell Res* 2012; **318**: 1577–85.
- Friedl P, Wolf K. Tumor-cell invasion and migration: diversity and escape mechanism. *Nat Rev Cancer* 2003; **3**: 362–74.
- Limame R, Wouters A, Pauwels B *et al*. comparative analysis of dynamic cell viability, migration and invasion assessments by novel real-time technology and classic endpoint assays. *PLoS One* 2012; **7**: e46536.
- Ampuja M, Jokimäki R, Juuti-Uusitalo K *et al*. BMP4 inhibits the proliferation of breast cancer cells and induces an MMP-dependent migratory phenotype in MDA-MB-231 cells in 3D environment. *BMC Cancer* 2013; **13**: 429.
- Deakin NO, Turner CE. Distinct roles for paxillin and hic-5 in regulating breast cancer cell morphology, invasion, and metastasis. *Mol Biol Cell* 2011; **22**: 327–41.
- Matsui T, Takano M, Yoshida K *et al*. Neural stem cells directly differentiated from partially reprogrammed fibroblasts rapidly acquire gliogenic competency. *Stem Cells* 2012; **30**: 1109–19.

- 25 Lin S, Fonteno S, Satish S, Bhanu B, Talbot P. Video bioinformatics analysis of human embryonic stem cell colony growth. *J Vis Exp* 2010; **39**: 1933.
- 26 Paraic AK, Genee YL, Connie AM, Neve RM, Semeiks JR, Spellman PT. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol* 2007; **1**: 84–96.
- 27 Bissell DM, Hammaker LE, Meyer UA. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. I Functional studies. *J Cell Biol* 1973; **59**: 722–32.
- 28 Bissell DM, Tilles JG. Morphology and function of cells of human embryonic liver in monolayer culture. *J Cell Biol* 1971; **50**: 222–31.
- 29 Bissell MJ. The differentiated state of normal and malignant cells or how to define a “normal” cell in culture. *Int Rev Cytol* 1981; **70**: 27–100.
- 30 Emerman JT, Pitelka DR. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In vitro* 1977; **13**: 316–28.
- 31 Celli JP, Rizvi I, Evans CL, Abu-Yousif AO, Hasan T. Quantitative imaging reveals heterogeneous growth dynamics and treatment-dependent residual tumor distributions in a three-dimensional ovarian cancer model. *J Biomed Opt* 2010; **15**: 051603.
- 32 dit Faute MA, Laurent L, Ploton D, Poupon MF, Jardillier JC, Bobichon H. Distinctive alterations of invasiveness, drug resistance and cell-cell organization in 3D-cultures of MCF-7, a human breast cancer cell line, and its multi-drug resistant variant. *Clin Exp Metastasis* 2002; **19**: 161–8.
- 33 Hiroto M, Xlaoblaio W, Eisuke M, Mekada E. Integrin signal masks growth-promotion activity of HB-EGF in monolayer cell cultures. *J Cell Sci* 2009; **122**: 4277–86.
- 34 Härmä V, Schukov HP, Happonen A et al. Quantification of dynamic morphological drug responses in 3D organotypic cell cultures by automated image analysis. *PLoS One* 2014; **9**: e96426.
- 35 Härmä V, Virtanen J, Mäkelä R et al. A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses. *PLoS One* 2010; **5**: 10431.
- 36 Rondón-Lagos M, Di Cantogno LV, Marchiò C et al. Differences and homologies of chromosomal alterations within and between breast cancer cell lines: a clustering analysis. *Mol Cytogenet* 2014; **7**: 1–14.

Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Material and Methods. Primary culture of human breast cancer tissue assay development. Immunofluorescence microscopy. Cell survival assay.

Video S1a. BT474 spheroids on Nanoculture Plates (NCP) with no drug.

Video S1b. BT474 spheroids on Nanoculture Plates (NCP) with 0.1 µg/mL trastuzumab.

Video S1c. BT474 spheroids on Nanoculture Plates (NCP) with 1 µg/mL trastuzumab.

Video S1d. BT474 spheroids on Nanoculture Plates (NCP) with 10 µg/mL trastuzumab.

Video S1e. BT474 spheroids on Nanoculture Plates (NCP) with 0.06 µg/mL paclitaxel.

Video S1f. BT474 spheroids on Nanoculture Plates (NCP) with 0.6 µg/mL paclitaxel.1

Video S1g. BT474 spheroids on Nanoculture Plates (NCP) with 6 µg/mL paclitaxel.

Video S2a. T47D spheroids on Nanoculture Plates (NCP) with no drug.

Video S2b. T47D spheroids on Nanoculture Plates (NCP) with 10 µg/mL trastuzumab.

Video S2c. T47D spheroids on Nanoculture Plates (NCP) with 6 µg/mL paclitaxel.

Video S3a. Human breast cancer primary cells B58 spheroids on Nanoculture Plates (NCP) with no drug.

Video S3b. Human breast cancer primary cells B58 spheroids on Nanoculture Plates (NCP) with 6 µg/mL paclitaxel.

Video S3c. Human breast cancer primary cells B58 spheroids on Nanoculture Plates (NCP) with no drug.

Video S3d. Human breast cancer primary cells B58 spheroids on Nanoculture Plates (NCP) with 6 µg/mL paclitaxel.