

# 3D matrix-based cell cultures: Automated analysis of tumor cell survival and proliferation

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**Abstract.** Three-dimensional *ex vivo* cell cultures mimic physiological *in vivo* growth conditions thereby significantly contributing to our understanding of tumor cell growth and survival, therapy resistance and identification of novel potent cancer targets. In the present study, we describe advanced three-dimensional cell culture methodology for investigating cellular survival and proliferation in human carcinoma cells after cancer therapy including molecular therapeutics. Single cells are embedded into laminin-rich extracellular matrix and can be treated with cytotoxic drugs, ionizing or UV radiation or any other substance of interest when consolidated and approximating *in vivo* morphology. Subsequently, cells are allowed to grow for automated determination of clonogenic survival (colony number) or proliferation (colony size). The entire protocol of 3D cell plating takes ~1 h working time and pursues for ~7 days before evaluation. This newly developed method broadens the spectrum of exploration of malignant tumors and other diseases and enables the obtainment of more reliable data on cancer treatment efficacy.

## Introduction

The role of targeted therapies in medical oncology has tremendously increased over the last ten years. A high number

of novel molecular substances have already been approved for clinical use and several compounds are in ongoing trials at present (1,2). Despite some very successful therapeutics like the tyrosine kinase inhibitor imatinib, which greatly ameliorated the outcome of patients suffering from chronic myelogenous leukemia (3), several of the molecular drugs have not met the expectations from preclinical data when applied clinically. We reason that one cause for this discrepancy could be that many drugs are tested under non-physiological two-dimensional (2D) cell culture conditions not sufficiently reflecting the microenvironment *in vivo*.

Three-dimensional (3D) cell culture models are in use for several decades now. Amongst scientists from various fields of biology and medicine, the culturing of cells in three dimensions opened new avenues of experimentation and thinking. Aside from its potential for tissues engineering, our understanding of cell biology has reached a new dimension ranging from gene expression to protein-protein interactions and signal transduction. The groundbreaking work of Bissell and co-workers and many others strikingly exhibited the essence of 3D growth conditions for single cells and higher order multicellular organisms (4-7).

Today, a large body of literature evidently demonstrates that the response of 3D grown cells to external stress and stimuli such as drug treatment or exposure to ionizing radiation more reliably reflects the cell response *in vivo* than the results obtained under 2D cell monolayer growth conditions (4,8-16). This effect could be due to both, the change in morphology and the activation of integrins and other cell adhesion receptors by binding to the ECM components, which strongly impact on cell behavior, functionality, gene and protein expression, protein-protein interactions, signal transduction and cellular sensitivity to cytotoxic stress (7,15,17-28). For *in vitro* investigation, cell phenotype and molecular processes can be conserved in 3D ECM-based scaffolds. This understanding gains particular relevance in the field of translational research. An example of even higher clinical relevance is a whole genome gene expres-

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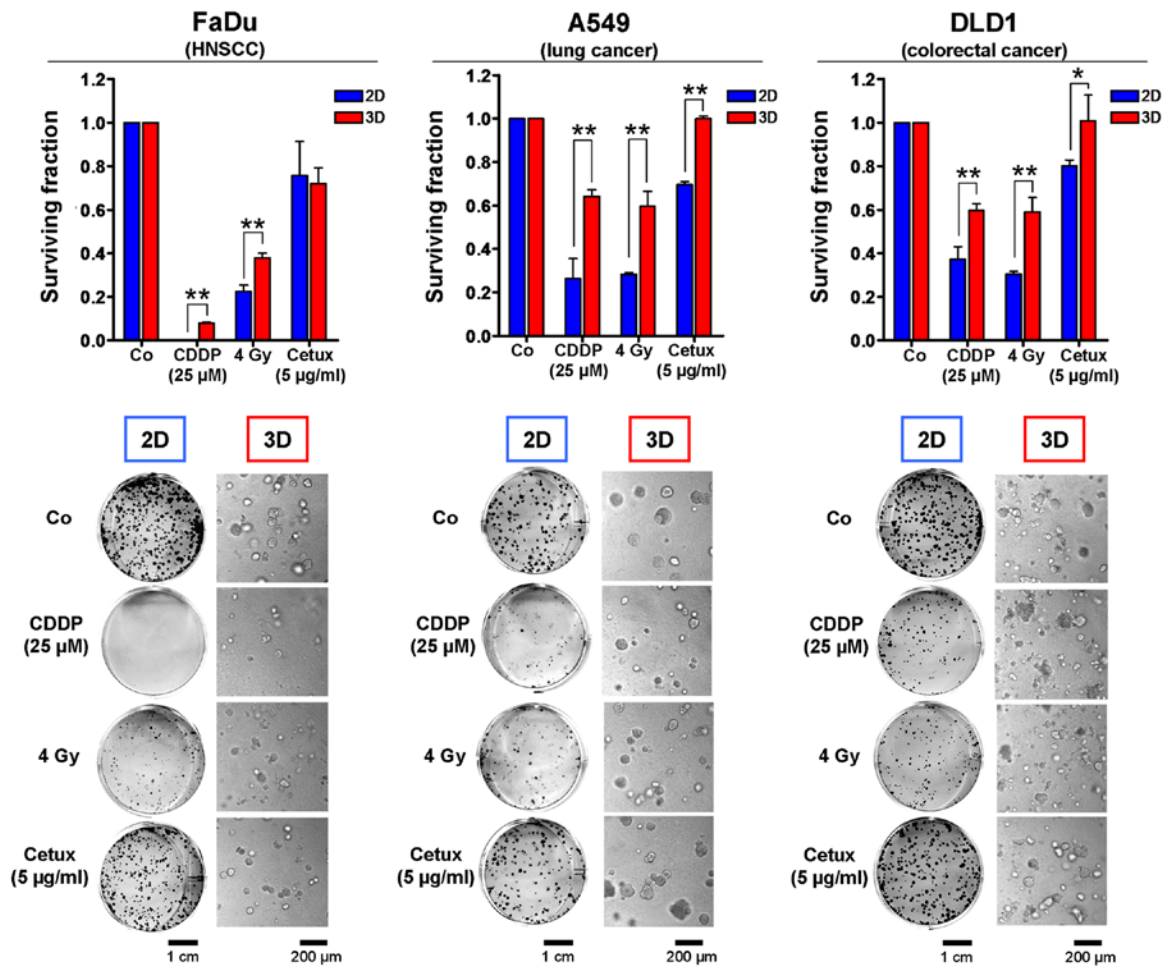


Figure 1. Cell culture conditions affect the tumor cell resistance to irradiation, chemotherapy and molecular therapeutics. Clonogenic cell survival data of a variety of human carcinoma cell lines treated with different clinically applied therapeutics as examples for anticipated results. Two (2D)- or three (3D)-dimensionally grown FaDu squamous cell carcinoma, A549 lung carcinoma and DLD1 colorectal carcinoma cells were treated either with cisplatin (CDDP), X-rays (4 Gy single dose) or the anti-EGFR antibody cetuximab. Images illustrate characteristic growth of cell colonies in 2D and 3D. Data show mean  $\pm$  SD (n=3; t-test; \*P<0.05, \*\*P<0.01).

sion analysis of 3D grown human breast cancer cell lines, which was elegantly used to demonstrate predictive power for the probability of relapse and overall survival of breast cancer patients (12,22).

By keeping in mind the heterogeneous distribution and expression patterns of ECM proteins in the different types of human malignancies, cell phenotypes of normal epithelial cells and cancer cells can be reproducibly maintained or restored by culturing them in laminin-rich basement membrane extracellular matrix (IrECM; Matrigel) (7,29). Either embedded or 'on top' with subsequent IrECM overlay, the IrECM isolated from the Engelbreth-Holm-Swarm mouse sarcoma provides a broad spectrum of applications for 3D cell investigations including measurement of apoptosis, cell proliferation, malignant transformation and differentiation. A variety of published protocols explains how cells can be isolated from IrECM gels for protein expression and functional exploration or examined *in situ* using microscopy on living cells or histology (immunohistochemistry, immunofluorescence) on fixed cells, organotypic cell cultures or tissues (23,29).

Cell survival *in vitro* is often measured in terms of apoptosis, dye exclusion or proliferation. Although more time

consuming, the colony forming assay has been shown to reliably determine tumor cell kill and reflect tumor control, whereas proliferation assays are used to explore tumor growth delay (30,31). Consequently, the colony forming assay is the gold standard for all disciplines for evaluating dose-effect relationships between e.g. drug concentration or radiation dose and cell survival (32).

However, to date, there is no existing assay to determine clonogenic cell survival as well as tumor proliferation under 3D cell culture conditions in a large scale for drug efficacy testing. On this basis, in the present study, we describe a high-throughput 3D IrECM based cell culture technique that greatly broadens the spectrum of already existing 3D cell culture protocols and enables a robust, reliable and reproducible analysis of the cancer cell response to cytotoxic drugs, targeted therapeutics or different kinds of radiation.

## Materials and methods

**Cell culture.** FaDu, A549 and DLD1 carcinoma cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The origin and stability of the cells

were routinely monitored by short tandem repeat analysis (microsatellites). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories GmbH, Coelbe, Germany) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories) and 1% non-essential amino acids (PAA Laboratories) at 37°C in a humidified atmosphere containing 7% CO<sub>2</sub>. For all experiments asynchronously growing cell cultures were used.

**Radiation exposure.** Irradiation (X-rays, 200 kV, 20 mA) was performed at room temperature using a Yxlon Y.TU 320 (Yxlon International CT Development GmbH, Hattingen, Germany) containing a 0.5-mm copper filter. For measurement of the absorbed dose a Duplex dosimeter (PTW Freiburg GmbH, Freiburg, Germany) was used. The dose-rate was ~1.3 Gy/min and applied doses ranged from 0 to 4 Gy.

**2D colony formation assay.** Asynchronously growing cells were trypsinized, counted using a Neubauer counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and plated as single cells in 6-well cell culture plates. After 24 h, cells were irradiated with 4 Gy or treated with cisplatin (25 µM) or cetuximab (5 µg/ml; Merck, Darmstadt, Germany) or left untreated. After 1 h cells were washed with 1X PBS to remove cisplatin from the cell culture medium. For determination of long-term survival cells were cultured for 8 days (A549, DLD1) or 11 days (FaDu) enabling colony growth. After fixation with 80% ethanol cells were stained with Coomassie blue (Merck). Counting of cell colonies with >50 cells was performed using a Stemi 2000 microscope (Carl Zeiss, Jena, Germany). Surviving fractions were calculated as follows: numbers of colonies formed/[numbers of cells plated (irradiated) x plating efficiency (unirradiated)]. Each point on survival curves represents the mean surviving fraction from at least three independent experiments.

**3D colony formation assay.** Asynchronously growing cells were trypsinized, counted and mixed with cell culture medium containing 0.5 mg/ml IrECM (cat. no. 354248; BD Biosciences, Heidelberg, Germany). Then, 100 µl of this mixture was placed in 96-well plates precoated with 50 µl of 1% agarose. After 2 h, the cell-IrECM layer was covered with 100 µl of cell culture medium. To prevent evaporation of medium, circumjacent wells were filled with 1X PBS (Fig. 1). After 24 h cells were irradiated with 4 Gy or treated with cisplatin (25 µM) or cetuximab (5 µg/ml) or left untreated similar to 2D cell culture conditions. To withdraw cisplatin from the cell culture, medium was carefully removed without touching the cell-IrECM layer and new cell culture medium was added. This step was repeated five times. Cells were cultured for 8 days (A549, DLD1) or 11 days (FaDu). Cell clusters (with the minimum size of a cell cluster containing 50 cells) were either counted microscopically without staining using a Axiovert 25 with a 2.5x objective (Carl Zeiss) or evaluated automatically as described below.

**Automated evaluation of colony number and size.** For automated analysis of survival and proliferation, each well was imaged in at least 7 different Z-levels using an Axio Observer microscope with a 2.5x objective (Carl Zeiss). ImageJ/Fiji (33)

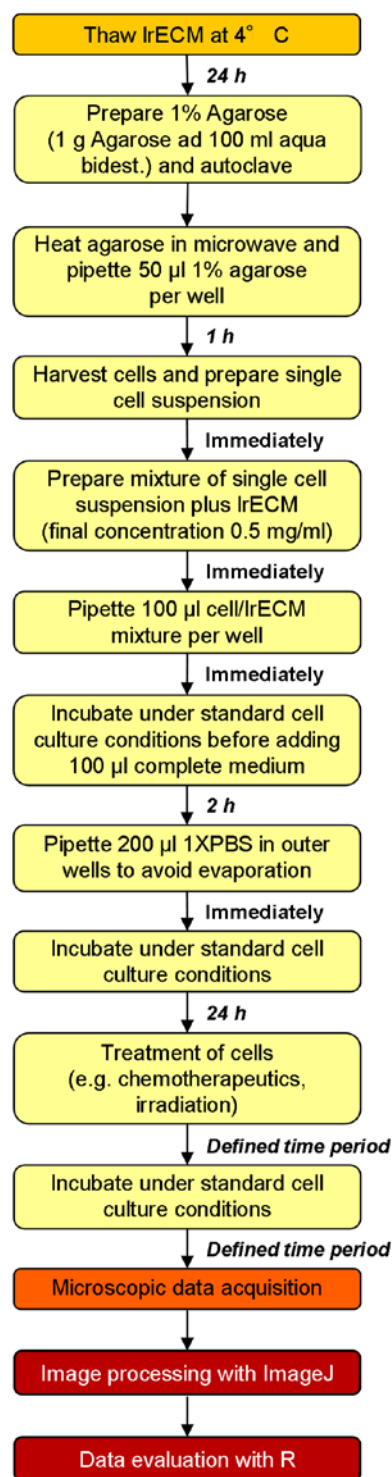


Figure 2. Summary of steps for 3D cell plating and analysis. Detailed workflow of experimental setup including incubation times. See also Figs. 3 and 5 for more information.

was used for image processing and an example ImageJ macro is shown in Table I. Briefly, focus stacking was applied to the Z-level images to yield a single clear image of all 3D colonies. Further processing included background subtraction, median filtering and thresholding steps. A watershed algorithm was used to separate overlapping colonies, and automatic colony counting followed. Tables of object sizes and numbers are written to disk. Overlays of the microscopic images and the

Table I. Example ImageJ macro to be used on a directory with multiple subdirectories containing images.

Steps	
Step 1	<pre>//ATTENTION: This macro will close all other open images in ImageJ Dialog.create("ATTENTION"); Dialog.addMessage("This macro will close all other open images in ImageJ/Fiji!! Please press cancel if there is any unsaved data"); Dialog.show();</pre>
Step 2	<pre>//Chose directory containing the image subdirectories dir = getDirectory("Choose a Directory "); count = 1; list = getFileList (dir);</pre>
Step 3	<pre>//Chose minimum colony size Dialog.create("Minimum colony size"); Dialog.addMessage("Please specify the minimum colony size (area) for counting (in pixels, smaller structures will be ignored)"); Dialog.addNumber("Minimum colony size", 600, 0, 5, "area pixels") Dialog.show(); MinColonySize = Dialog.getNumber();</pre>
Step 4	<pre>//Loop through directories for (i=0; i&lt;list.length; i++) { if (endsWith(list[i], "/")) { //Get files in directory files = getFileList(""+dir+list[i]);</pre>
Step 5	<pre>//load images and perform focus stacking run("Image Sequence...", "open=" + dir + list[i] + files[0] + " sort"); run("Extended Depth of Field (Easy mode)...", "quality='0' topology='0' show-topology='off' show-view='off');</pre>
Step 6	<pre>//Wait for output to open while(!isOpen("Output")) { wait(50);} wait(1000); //Just to make sure not too early</pre>
Step 7	<pre>//Select stacked output selectImage("Output"); rename("OStack"); run("Duplicate...", " ");</pre>
Step 8	<pre>//Substract background, filter and do segmentation run("8-bit"); run("Subtract Background...", "rolling=50 light"); run("Median...", "radius=3"); run("Auto Threshold", "method=Default white"); run("Convert to Mask"); run("Watershed"); rename("Segmented");</pre>
Step 9	<pre>//Count Colonies run("Analyze Particles...", "size=MinColonySize -Infinity circularity=0.00-1.00 show=[Overlay Outlines] display clear");</pre>

Table I. Continued.

Steps	
Step 10	<pre>//Save overlay image to disk selectWindow("OStack"); run("Select All"); run("Copy");selectWindow("Segmented"); run("Paste"); run("Invert"); setFont("SansSerif", 32); setColor(120,120,120); setJustification("left"); drawString("Min. Colony size: "+ MinColonySize, 10, 50); saveAs("PNG", dir + "segmented" + files[0]);</pre>
Step 11	<pre>//Save results to disk selectWindow("Results"); saveAs("Results", dir+ substring(list[i],0,lengthOf(list[i])-1) + ".csv");</pre>
Step 12	<pre>//Close all windows close("*");}}</pre>

Each subdirectory should contain multiple images of one well at various levels. Images will be focus stacked and cell colonies will be counted. Output will be a text file for each directory with information on all colonies identified and an image showing the counted colonies for quality check.

Table II. Example R code to be used to analyze the data.

Steps	
Step 1	<pre>#Set to directory containing the ImageJ output setwd("C:/Users/xx/Images") #Adjust this to the directory containing the image subfolders</pre>
Step 2	<pre>library("ggplot2") require(plyr)</pre>
Step 3	<pre>#load data #get names of csv files, read and add each filename to the dataframe files &lt;- dir(pattern = "*.csv") data &lt;- read.csv(files[1],header = TRUE) data\$file &lt;- files[1] for (i in 2:length(files)){ a &lt;- read.csv(files[i],header = TRUE) a\$file &lt;- files[i] data &lt;- rbind(a,data)}</pre>
Step 4	<pre>#create and output area histograms p &lt;- ggplot(data, aes(x=Area)) + geom_histogram() +scale_x_log10() + facet_wrap(~file) pdf("histograms.pdf", , width=8, height=10) print(p) dev.off()</pre>
Step 5	<pre>#summarize data for each filename (corresponding to each well) and write csv file resultdata &lt;- ddply(data, .(file), summarize, ColonyNumber=length(Area) , MeanColonyArea=mean(Area), TotalColonyArea = sum(Area)) write.csv(resultdata, file = "SummaryCounting.txt")</pre>

The data are loaded to R and area histograms are created.

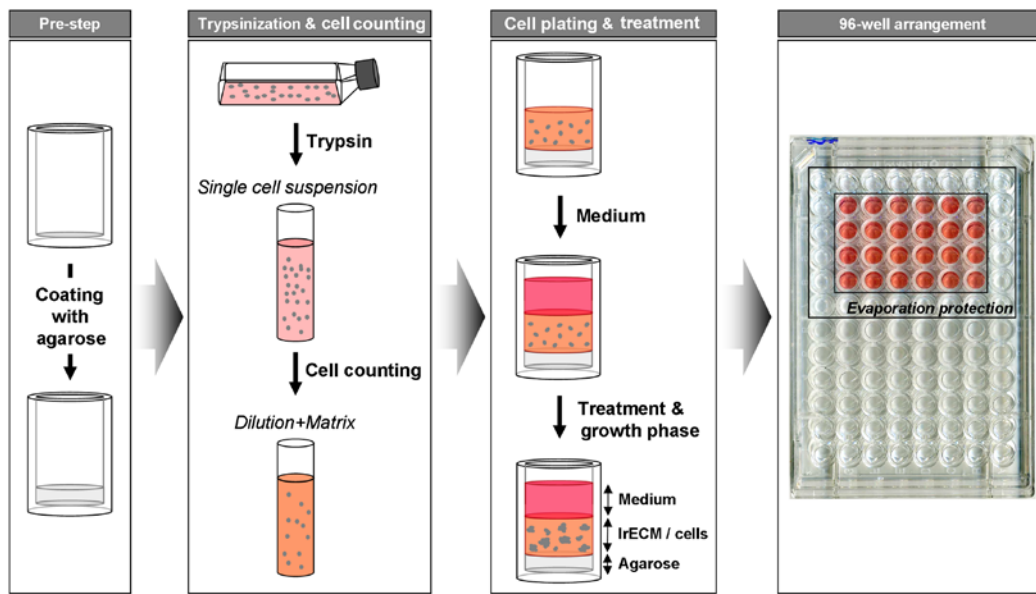


Figure 3. Procedure of 96-well preparation and cell plating into 3D IrECM. To prevent cell adhesion to bottom, wells are coated with agarose. Single cell suspension is prepared from permanent cell culture, diluted with IrECM and pipetted on top of polymerized agarose. Medium is added after polymerization of cell-IrECM mixture. Upon treatment, cell colonies grown from single cells can be counted and/or analyzed microscopically.

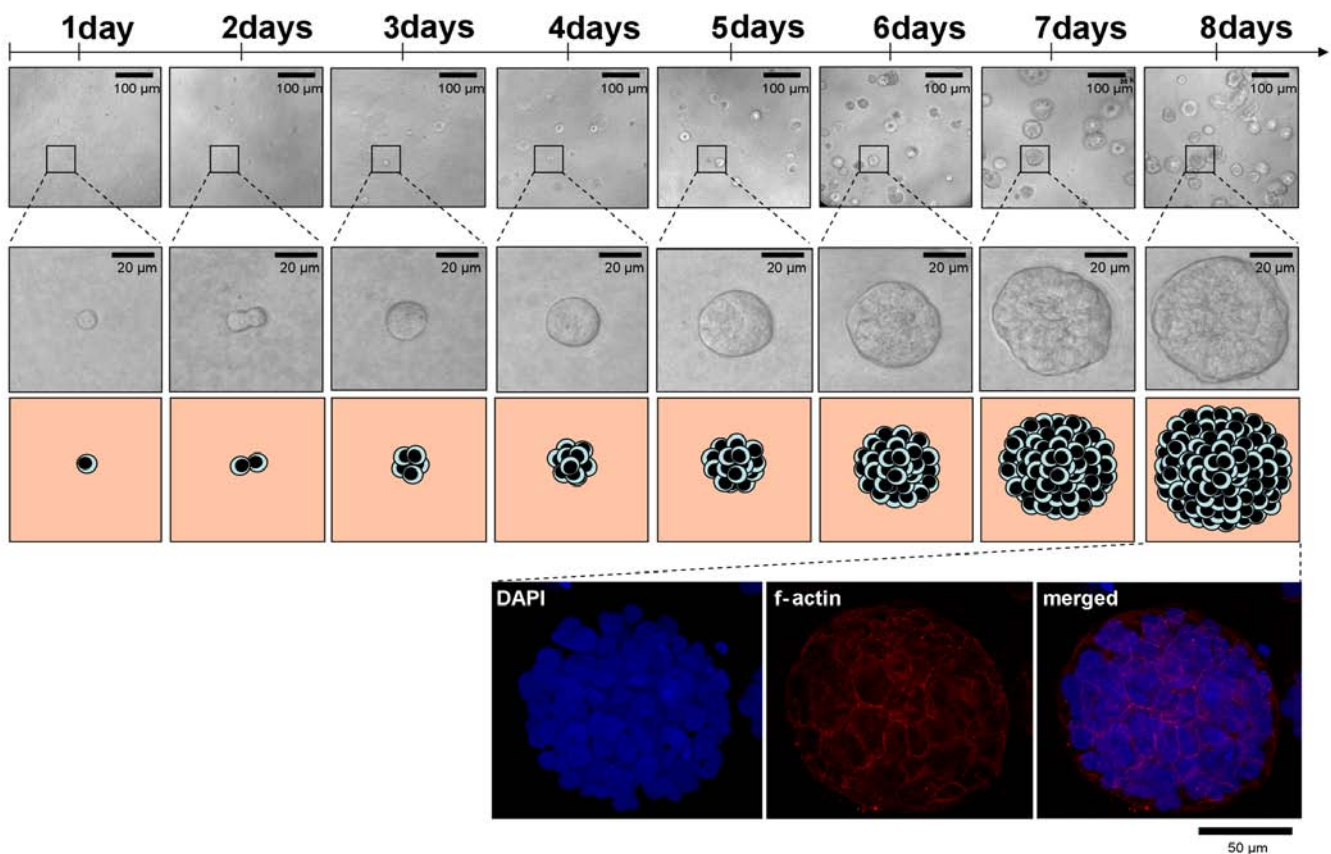


Figure 4. Colonies in 3D IrECM continuously grow out of one single cell. Representative cell colony growth over 8 days illustrated by microscopic pictures and cartoons. Securing a more than 50 cell stage of a single cell colony was achieved by DAPI staining (optional f-actin staining with phalloidin).

segmentation are also saved for quality review. The resulting tables can be further summarized and analyzed using R (34). The example scripts (ImageJ: Table I; R: Table II) automatically process images from multiple wells. The R script generates a

histogram of colony size for each well and a summary result table of all wells imaged containing the number of colonies, the average colony area and the total colony area as a measurement of proliferation.



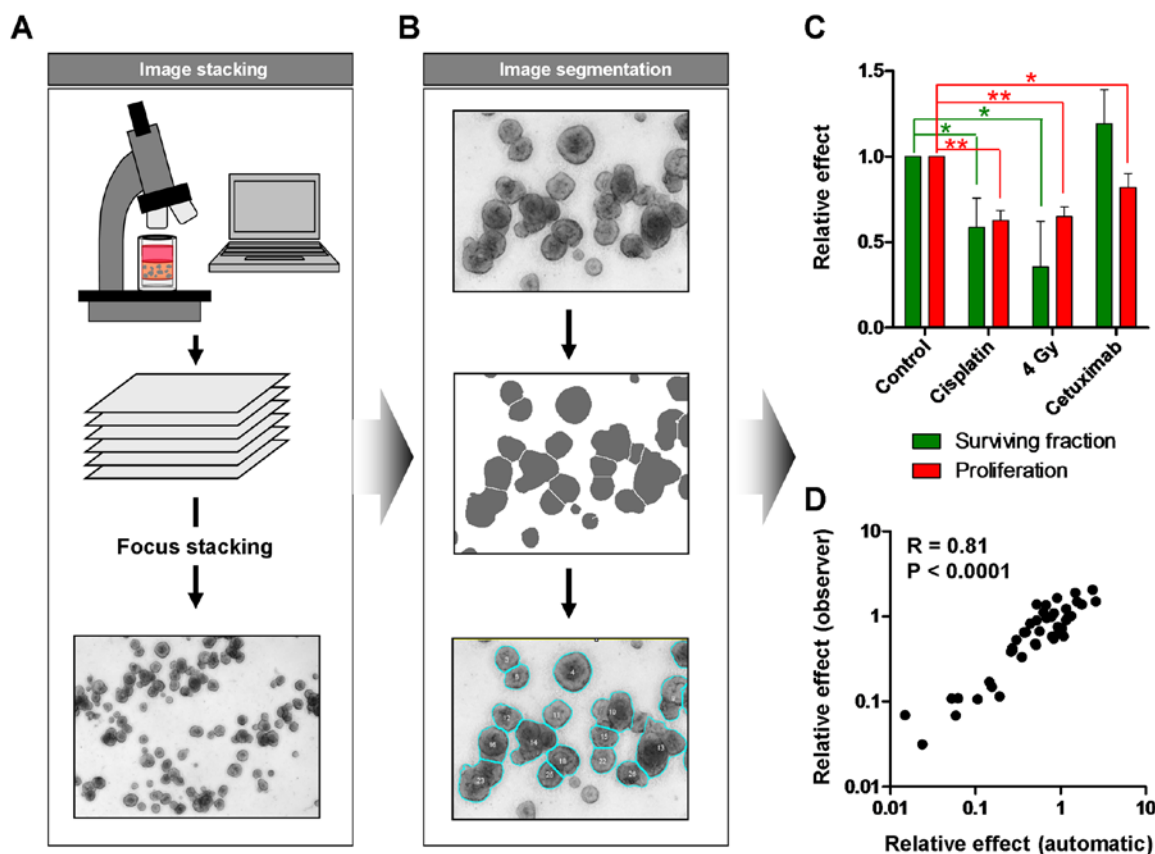


Figure 5. Automated analysis of 3D colony formation assay can differentiate between effects on survival and proliferation. (A) To account for limited field depth when imaging 3D cell cultures, multiple Z-levels are recorded and merged using an algorithm that preserves the sharpness of each plane (focus stacking). (B) The resulting image is post-processed and segmented to delineate the colonies. (C) Colonies are automatically counted followed by colony size evaluation as surrogate for cell proliferation. Data show mean  $\pm$  SD (n=3; t-test; \*P<0.05, \*\*P<0.01). (D) Comparative analysis of automatically measured colonies and manually measured colonies including correlation calculation (R=0.81; P<0.0001).

## Results and Discussion

In the present study, we describe a novel method to measure automatically clonogenic survival and proliferation of cells in a 3D matrix consisting of lrECM which has been reported to mimic physiologic *in vivo* growth conditions in a better way than conventional 2D cell culture plastic (4-11,14,16). Importantly, this approach can also be used in a high-throughput setting. According to previous data, we found that the response of all three tested human carcinoma cell lines exposed to the chemotherapeutic drug cisplatin (CDDP) or to X-ray irradiation was affected by the growth conditions with cells being significantly more resistant when cultured in 3D (Fig. 1) (9,11,20,24,35). This cell adhesion-mediated radioresistance and therapy resistance might result from a multitude of cellular processes including differences in transcriptional, translational, post-translational processes and signal transduction (8-11,22,36). Not surprising and particularly alerting with regard to molecular drug efficacy is that molecular compounds like the EGFR inhibitor cetuximab are also less effective under 3D growth conditions and that this cellular drug response correlates more closely with *in vivo* results (Fig. 1) (9-11,37). These data confirm the necessity to test targeted substances and more conventional therapeutics in a 3D matrix-based *in vitro* assay prior to animal studies to minimize costs, time and effort.

The workflow of plating and treatment of cells for the 3D clonogenic assay is depicted in Figs. 2 and 3. Agarose, cell/lrECM mixture and medium can be applied with a multi-channel pipette allowing time-efficient plating for large-scale analysis. Another advantage over most of the existing matrix-based 3D methods is that the lrECM solution with the concentration of 0.5 mg/ml can be produced with pre-heated medium (37°C) and processed at room temperature for at least 30 min without becoming solid. Therefore, cells do not have to be cooled down which likely provoke a cold stress response and perturb molecular processes (38). To assess the cell number per colony and proliferation of cells embedded into lrECM, we evaluated the number of grown A549 cells over a period of 8 days microscopically (Fig. 4). Phase contrast microscopy and DAPI/f-Actin staining revealed similar proliferation rates of this cell line in a 3D matrix in comparison to 2D monolayer cell cultures (~22 h according to ATCC) with doubling times of about 24 h after a lag phase of 1 day. Importantly, at the time of treatment (1 day after plating), 3D cell cultures are still in the single cell status, a key requirement to measure clonogenic survival (Fig. 4) (30-32).

Manually counting of colonies is a time-consuming and error-prone process. Therefore, automated evaluation can reduce the working time and improve the inter-observer reliability and validity of data. As the colonies are in a 3D matrix, we took images of the wells in different Z-levels and

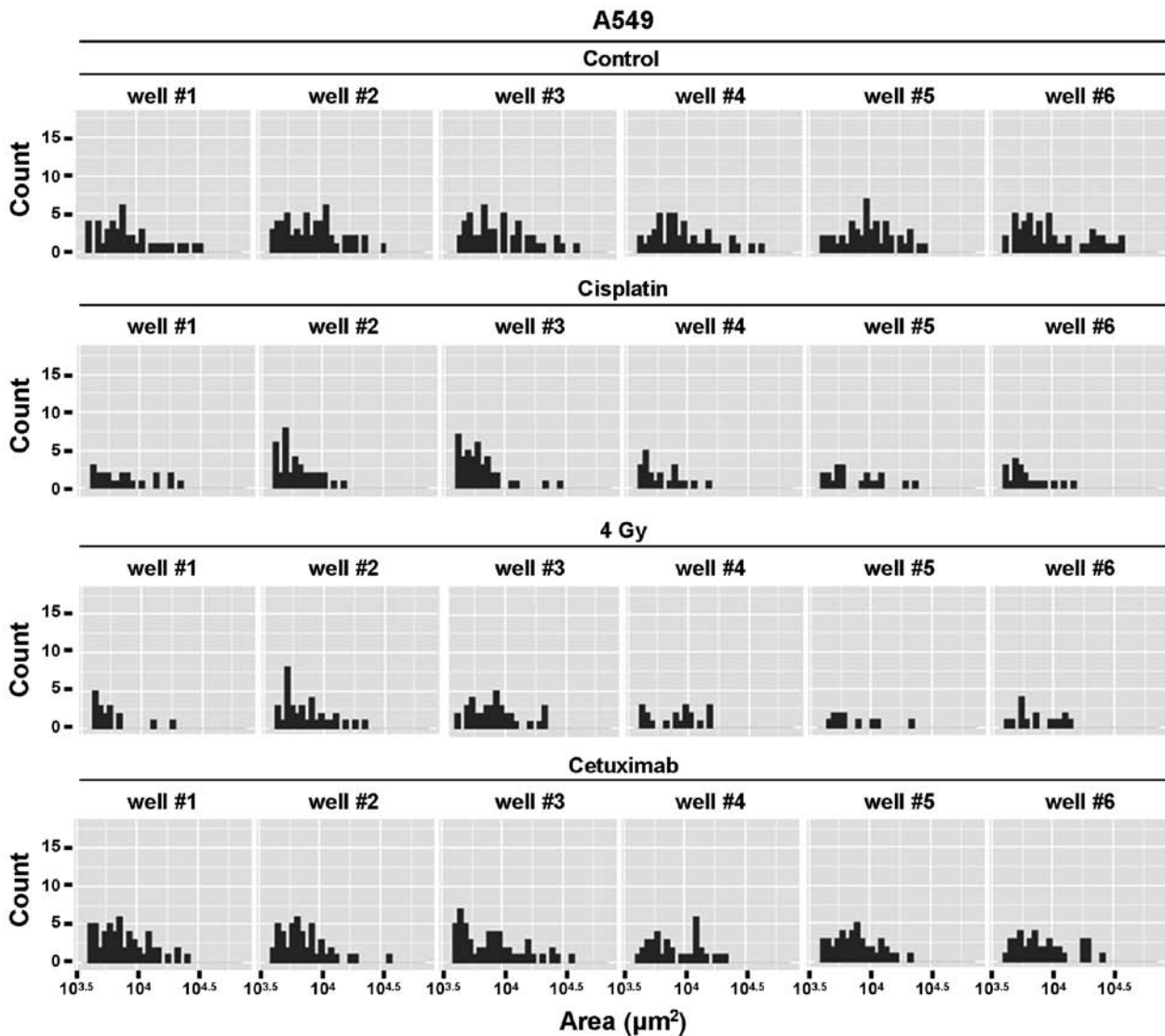


Figure 6. Detailed data analysis can reveal effects of cancer treatment on distribution of measured colony sizes on a single-cell-base. With the described method, the size of every single colony under each treatment condition is recorded and the distribution can be analyzed and illustrated with R. Histograms are plotted for each well separately showing the number of colonies (colony count) with a specific colony size (colony area).

performed focus stacking to create a clear image of all colonies (Fig. 5A). After image segmentation, colony number and size were determined (Fig. 5B) enabling the evaluation of the specific treatment effects on tumor cell proliferation as well as on clonogenic survival (Fig. 5C). While irradiation or cisplatin treatment reduced both, the size of the colonies and the colony number, cetuximab mainly affected the tumor growth and had no significant impact on cell survival (Fig. 5C). A comparison with manually counted results showed an excellent correlation ( $R=0.81$ ) indicating a high reliability of the obtained data (Fig. 5D).

Considering the heterogeneity in human tumors and the role of cancer stem cells for therapy resistance (39), analysis on a single cell base can be crucial to evaluate the potential of targeted therapeutics. With the described technique the size and distribution of every single colony (which grows out of one single cell) could easily be determined and plotted in a histogram (Fig. 6). As shown in Fig. 6, control cell cultures had a wide spectrum of colony sizes with several small and

medium-sized but also few larger colonies. In contrast, after exposure to cisplatin, ionizing radiation or cetuximab the distribution shifted to the left resulting in an overall decrease of colony size. These data could give valuable information about the different treatment effects in a tumor cell population.

In summary, the described protocol is a cost and time-efficient method to analyze the tumor response to cancer therapy in a more physiologic cell culture model. Taking into account that 3D IrECM based assays have been shown to reflect the *in vivo* conditions more reliably than 2D monolayer cells, it would be beneficial to employ this technique in a large-scale evaluation of molecular compounds prior to *in vivo* studies.

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