A Simple and Cost Efficient Method to Avoid Unequal Evaporation in Cellular Screening Assays, Which Restores Cellular Metabolic Activity

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Abstract

Spheroid based cellular screening approaches represent a highly physiologic experimental setup to identify potential novel chemical compounds in cancer research. Increasing numbers of chemical compound libraries are available, which offer straightforward development of preclinical drug screening assays for academic research. Cellular screening experiments are often performed in 96 well plates in a small culture volume for elongated time periods; therefore, growth medium volume decrease due to evaporation poses a problem. We show here that uneven loss of growth medium depending on the microwell position on the plate (edge and corner versus central positions) led to significantly different readouts in a cell metabolism assay (alamarBlue®) as indicator for cellular health and proliferation. These effects are due to both an increase in dye concentration and reduced cellular metabolic activity owing to the decrease in culture volume. The incubation of the 96 well plates in cheap, custom-made humidity chambers almost abolished the loss of liquid and restored equal growth medium volumes in all wells after prolonged culture. This led to evenly distributed alamarBlue® fluorescence intensity values on the multiwall-plate and warrants proper identification of effective compounds in screening assays.

Keywords: cellular based screening, three-dimensional cell culture, spheroid, alamar blue assay, evaporation, humidity chamber

Introduction

The first round of cellular based preclinical screening for potential anti-cancer drugs mostly relies on cell culture experiments in plastic plates. Cell culture on two-dimensional (2D) surfaces has provided groundbreaking insights into basic cell biology and tumorigenesis [1-3]. However, under these conditions many physiological parameters of organs or tumors such as tissue architecture, cell to cell – and cell to matrix interaction, mechanical properties and biochemical networks are lost. Cells grown in three-dimensional (3D) scaffolds or as 3D aggregates (multicellular spheroids) much better recapitulate the *in vivo* situation of tumors [4-9]. An important aspect is that drug response and drug and radiation resistance characteristics of multicellular tumor spheroids closely match those seen *in vivo* [10]. Therefore, these preclinical models display a more appropriate physiological system to test potential therapeutic targets in initial cell based screening attempts. Furthermore, tumors and spheroids have in common that proliferating and quiescent cells, normoxic and hypoxic cells, as well as alive and dead cells [11] coexist. Consequently, overall cell proliferation is reduced in 3D as compared to 2D cultures and longer incubation times are needed to assess proliferation- and growth inhibiting or cytotoxic effects with commonly used cell metabolic activity assays such as the MTT or the alamarBlue® assay.

A convenient and widely used experimental system for screening of compounds in cell-based assays is the use of 96 well plates. One disadvantage of 96 well plates in these studies is liquid loss due to evaporation [12,13], especially during extended incubation periods exceeding 24 h.

Differential evaporation of water from outer to inner wells of the microwell plate is one major problem contributing to parabolic well-to-well growth patterns, which affected the results of routine growth inhibition studies of anticancer agents [14]. It has also been observed that evaporation of liquid of the outer wells resulted in increased absorbance in MTS assays compared to the inner wells of a 96 well plate [15].

In order to prevent evaporation of liquids, self-adhesive plate sealers were invented, which reduce the evaporating effect increasing from the center- toward the edge- and finally to the corner wells of a microplate [13]. However, evaluation of different commercially available plate sealers revealed severe drawbacks and categorized them into 2 classes: (1) plate sealers in which water retention is relatively low, but O_2 transfer is comparable to that of unsealed plates and (2) plate sealers in which volume preservation is comparably high, but O_2 exchange is slower [12]. Therefore, these technical aids do not fulfill the requirements for proper drug screening in 96 well plates. Some laboratories have overcome this issue by not using the outer wells for cell-based assays [15].

We initiated a cellular screen with the commercially available Prestwick Chemical Library®, which requires the use of the entire 96 well plate due to the predefined position of molecules from columns 2 to 11. For the screening of 1200 different compounds, multicellular spheroids were cultured in 96 well plates in a standard cell culture incubator for more than four days. Therefore, evaporation of liquid from corner and edge wells of the microwell plates reflects a major problem. We hypothesized that a humidity chamber inside a standard cell culture incubator would reduce this issue. Importantly, we could recapitulate the findings in literature concerning liquid evaporation using alamarBlue® as readout for cell metabolism, which showed significant differences between 96 well plates cultured outside or inside the humidity chamber in a standard cell culture incubator. Here, we demonstrate that evaporation was greatly overcome by using a humidity chamber, which significantly reduced liquid loss. The use of the humidity chamber restored the differential effects seen in culture outside the chamber.

Materials and Methods

Cell culture

Human colonic cancer cell lines LS 174T (ATCC# CL-188), HCT 116 (ATCC# CCL-247) and HT-29 (ATCC# HTB-38) were obtained from the American Type Culture Collection (ATCC®) and were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g/l), supplemented with 10% fetal calf serum, 2 mM L-Glutamine and antibiotics (60 mg/l penicillin, 100 mg/l streptomycin sulfate) at 37°C and 5% CO_2.
Spheroid formation was induced as described [16]. In brief, cells were washed with PBS and harvested by trypsinisation. 1000 cells were seeded in 200µl cell culture medium per well in a 96 well plate (round bottom, untreated, PAA). Spheroids were formed and grown in DMEM high glucose (4.5 g/l), supplemented with 5% fetal calf serum, 2 mM L-Glutamine and antibiotics (60 mg/l penicillin, 100 mg/l streptomycin sulfate) containing methylcellulose (0.3% final concentration). 96 well plates were cultivated inside or outside the humidity chamber (see below) in a cell culture incubator (Thermo Scientific, Heraeus CO_2 incubator BBD 6220) under standard conditions with 80% humidity, 5% CO_2 and 20% O_2.

Humidity chamber

Figure 1b represents a scheme of the humidity chamber, which consists of a standard and cheap plastic box (IKEA, box: 701.029.72, lid: 101.103.00) into which ventilation holes were drilled in the bottom for proper O_2 saturation and a non air-tight lid. A water reservoir, containing 200 ml sterile distilled H_2O, inside the chamber ensures humidified atmosphere. It is possible to incubate a stack of up to ten 96 well plates in the chamber. The plate stack is separated from the water reservoir by a perforated intermediate layer. The humidity chamber is 28 cm x 20 cm x 14 cm.

AlamarBlue® cell metabolic assay

AlamarBlue® assay (AbD Serotec, BUF012B) was performed according to the manufacturer's protocol. 1/10 of culture volume of alamarBlue® was added to each well of the 96 well plate. The plates were incubated for a maximum of 96 h inside or outside the humidity chamber at 37°C and 5% CO_2. Fluorescence intensity was measured at 530 nm excitation wavelength and 590 nm emission wavelength after 24 h, 48 h, 72 h and 96 h using Biotek® Synergy HT Photometer. [17]

Volume measurement

96 well plates containing 200 µl liquid per well were incubated inside and outside the humidity chamber in a standard cell culture incubator (Thermo Scientific, Heraeus CO_2 incubator BBD 6220) for 96 h at 5% CO_2 and 80% humidity. After 24 h, 48 h, 72 h and 96 h volume measurement was performed by taking up 200 µl of liquid with an adjustable pipette (Eppendorf, 20-200 µl, 2223 511.344), removing the air content in the pipette tip by adjusting the volume with the setting ring, documenting the exact value and returning the liquid back in the well.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel 2007 for Windows. Bar graphs created in Microsoft Excel are presented as mean ± standard deviation (SD). Boxplots were created in GraphPad Prism 4. The box represents the middle 50% of the data ranging from the lower quartile (Q1) to the upper quartile (Q3). The line within the box indicates the median (Q2) and the whiskers represent the lower and upper 25% of the data - the minimum and maximum values respectively. For statistical analysis Student's *t*-test (unpaired, two-tailed) was carried out.

Results

For screening purposes, spheroids were cultured in a standard cell culture incubator at 37°C, 5% CO_2 and 80% humidity for more than four days. Therefore, evaporation of liquid in corner and edge wells of 96 well plates reflects a major problem. A decrease in volume was already visible by eye after 12 h of culture. A severe reduction of volume at the corner wells was obvious and edge wells also showed decreased volume. We hypothesized that a humidity chamber inside a standard cell culture incubator would reduce this effect. To evaluate the decrease in volume at different well positions in 96 well plates in the incubator, the 96 well plates were classified in corner, edge and central wells. The results are presented according to the zonal segmentation as shown in Figure 1a. The layout of the humidity chamber is given in Figure 1b. Up to ten 96 well plates can be placed in the box simultaneously.

Three 96 well plates containing 200 µl liquid per each well were incubated inside and outside the humidity chamber in a standard cell culture incubator for four days. A time course for corner wells (Figure 2a), edge wells (Figure 2b) and central wells (Figure 2c), representing the decrease in volume over an incubation time from 0 h – 96 h, was performed and displayed differences in remaining liquid in classified wells between culture outside or inside the humidity chamber. After 96 h the volume decreased in corner wells outside the chamber to 74% and inside to 91%. 84% and 93% of the initial total volume (200 µl) remained in edge wells outside and inside the chamber, respectively. The volume in central wells was reduced to 93% (outside) and to 95% (inside). The differences in volume of corner wells, edge wells and central wells outside and inside the humidity chamber after 96 h (Figure 2d-f) were highly significant. A direct comparison of the remaining liquid in corner, edge and central wells of 96 well plates outside the chamber compared to equivalent wells inside the chamber after 96 h is given in Figure 2g.

Our main interest in cultivating spheroids in 96 well plates is to perform cell-based drug screening approaches in a 3D setting. The main readout of the initial screening round is cellular health as a function of metabolism. Therefore, the alamarBlue® assay, a non-toxic, well-characterized cell viability and proliferation indicator, was performed.

Metabolic activity of spheroids in 96 well plates was determined inside and outside the humidity chamber. HT-29 spheroids (initial cell number 1000 cells/spheroid) were cultured for five days. 24 h after spheroid formation alamarBlue® solution was added to each well and further incubated for 96 h. Thereafter, fluorescence intensity levels were measured. The difference in fluorescence intensity levels of corner wells (Figure 3a), edge wells (Figure 3b) and central wells (Figure 3c) incubated inside and outside the humidity chamber is shown. Corner, edge and central wells show significant differences between cultivation outside or inside the humidity chamber. These results could be interpreted in two ways: (a) either a real reduction of cell proliferation/health due to reduced volumes in the corner/edge wells occurred or (b) the data could result from different alamarBlue® dye concentration and changed fluorescence properties due to the loss of liquid. To test this evaporated volume in corner, edge and central wells in plates kept outside the chamber was refilled with aqua dest to equalize the volume of corresponding wells in plates inside the chamber and were re-measured.

The fluorescence values of the non-humidified plates could only be partially restored in the "outside" plates by addition of the lacking water as compared to the humidified wells (Figure 3d-f). This indicated that reduced medium volume has also an effect on cell viability/proliferation/metabolic activity. The differences after readjusting the volume were highly significant between edge ($p = 0.0004$) and central wells ($p = 0.0001$) of 96 well plates outside or inside the humidity chamber. A comparison of the alamarBlue® fluorescence intensities within the same plate between different well positions revealed significant differences between center, edge and corner wells (Figure 4 a) when cultivated outside the humidity chamber. Importantly, there was no significant difference in alamarBlue® fluorescence detectable any longer between edge and central wells when the plates were incubated inside the humidity chamber (Figure 4 b). However, the fluorescence in the corner wells was marginal but still significantly lower as compared to edge and center wells (Figure 4 b), indicating that the 4 corner wells per plate should not be included in the screening procedures. This experiment was repeated with LS 174T and HCT 116 colon cancer cell lines and showed similar results (data not shown). Taken together, we demonstrate that the use of simple, cheap and easy to set up humidity chambers reduce uneven evaporation in wells at different positions in 96 well plates and restore reliable alamarBlue® assay readouts, independent from the microwell position.

Discussion

Cells grown in three-dimensional (3D) scaffolds or as 3D aggregates (spheroids) are well suited for drug screening approaches in cancer research since they closely mirror the *in vivo* situation in many aspects [11]. However, incubation times have to be extended due to the reduced proliferation rates, in order to obtain significant results. The reduction of proliferation results from the simultaneous presence of proliferating, non-proliferating and dying or dead cells in these assays again closely reflecting the *in vivo* situation. The extended incubation period leads to the problem of greatly uneven distribution of evaporation in microwells dependent on their position in the multiwell plates. Our results confirm the findings of other studies, which reported uneven distributed liquid evaporation [13-15]. We show that the liquid loss varied between 7% and 26% depending on the well position, when cultured for 96 h. The corner wells lost most liquid, whereas in center wells only little liquid evaporated. This variation of evaporation was only between 5 to 9%, when the microplates were incorporated in the humidifying chamber. Differential liquid loss could affect cell viability of multicellular spheroids. Alternatively, different alamarBlue® readouts could simply be due to different indicator dye concentrations due to more or less evaporation in certain wells. For clarification, the evaporated volume was refilled by H_2O dest, which resulted in a reduction but not an elimination of the differences in fluorescent intensity levels of alamarBlue®. This indicated that (a) the alamarBlue® assay was prone to variations in growth medium concentrations due to different evaporation rates and (b) cellular health/proliferation/metabolism was also affected.

We conclude that loss of liquid due to evaporation in combination with variations in growth rates based on different growth medium osmolarity may lead to misinterpretation of efficiencies of tested molecules in a drug screening approach. A possible solution for the liquid loss in 96 well plates could be sealing with plate sealers, which showed no satisfying results [12]. Another possibility is not to use the edge/corner wells leading to a reduction from 96 to 60 test wells/plate (minus 37.5%). Consequently, space, economic and environmental aspects have to be considered in this case. As a third possibility we offer a straightforward and cost efficient method to avoid liquid loss by using custom-made humidifying boxes. We prove that the use of these chambers is sufficient to avoid extensive evaporation and to get evenly distributed spheroid metabolic activity in 92 wells of a 96-well plate. We propose to omit the 4 corner wells of a 96-well plate for cellular screening approaches, as these are still subjected to a small but significant loss of liquid even inside the humidity chamber, which perturbs the alarmarBlue® assay. Leaving out the corner wells still allows to include sufficient amounts of positive or negative controls per plate (e.g. using wells B1-G1 and B12-G12 for controls, compare Figure 1 a and the remaining 80 wells for the actual drug screening). Finally, there is no special equipment needed for the assay and the humidity chamber can be set up in less then 30 min using standard household plastic boxes.

The increasing availability of chemical compound libraries offers even smaller academic institutions to perform medium to high throughput cellular screens in specialized settings, not covered by big pharmaceutical industry screening programs. Our methodological improvement might be of relevance for these 3D cellular based drug-testing approaches.

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Figure Legends

Figure 1. *Schematic representation of a 96 well plate and the humidity chamber*. **(a)** Zonal segmentation of wells (corner, edge, central) of 96 well plate. **(b)** Outline of the humidity chamber - a plastic box with ventilation holes in the bottom for proper O_2 saturation and with a non air-tight lid. Humid atmosphere is ensured by a water reservoir inside the chamber. A perforated intermediate layer separates the stack of 96 well plates from the water reservoir. Dimensions of the chamber: length = 28 cm, width = 20 cm, height = 14 cm.

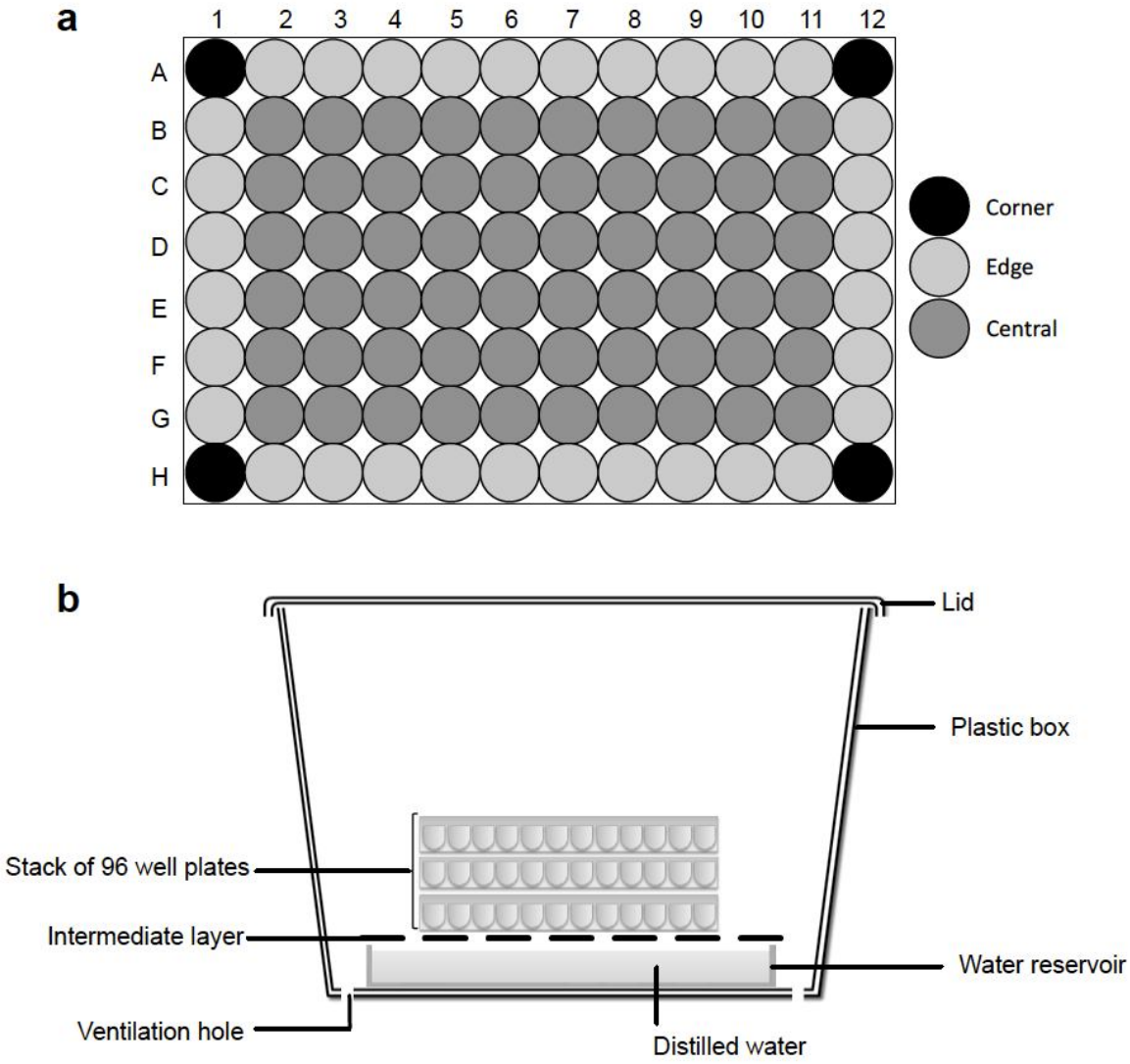

Figure 2. *Significant decrease in volume in corner and edge wells of 96 well plates.* Time dependent decrease of medium between 96 well plates incubated in the humidity chamber as compared to normal incubation. The volume decrease is shown for **(a)** corner wells (n = 4 per condition), **(b)** edge wells (n = 20 per condition) and **(c)** central wells (n = 14 per condition). Data are presented as mean ± standard deviation (SD). Volume of **(d)** corner wells (n = 4 per condition), **(e)** edge wells (n = 20 per condition) and **(f)** central wells (n = 14 per condition) of 96 well plates inside and outside the chamber was measured after 96 h showing a significant volume decrease between these two conditions. The boxes in the boxplots represent the middle 50% of the data ranging from the lower quartile (Q1) to the upper quartile (Q3). The line within the box indicates the median and the whiskers represent the lower and upper 25% of the data - the minimum and maximum values respectively. **(g)** Comparison of volume between corner, edge and central wells of 96 well plates inside and outside the chamber after 96 h. Data are presented as mean ± standard deviation (SD). P-values are indicated.

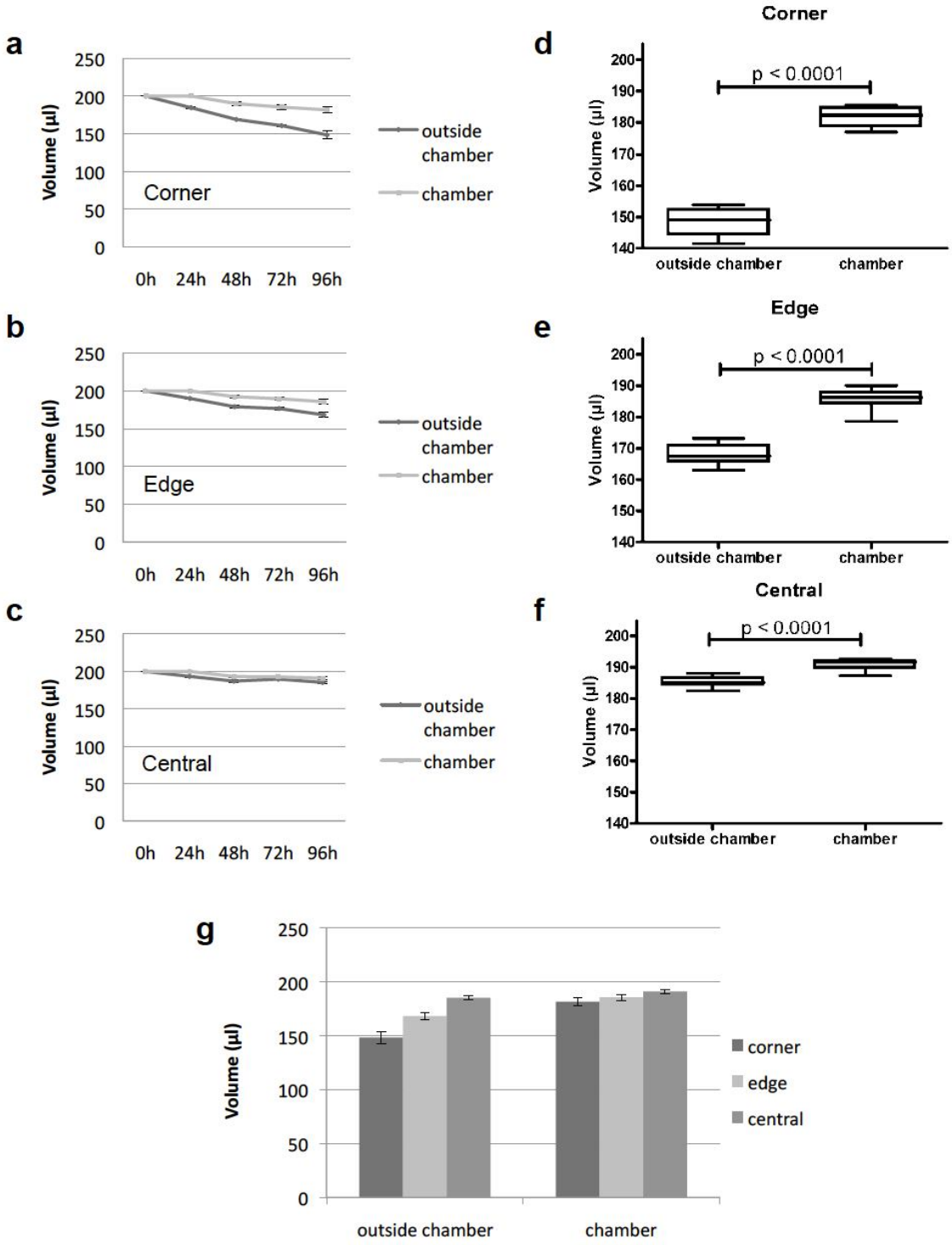

Figure 3. *alamarBlue® assay, indicating cell growth, displays significant difference between corner and edge wells inside and outside the chamber.* HT-29 spheroids were cultured in 200 µl cell culture medium in 96 well plates inside and outside the chamber. alamarBlue® assay was measured after 96 h. Boxplots represent the difference between **(a)** corner wells (n = 8 per condition), **(b)** edge wells (n = 32 per condition) and **(c)** central wells (n = 60 per condition) of 96 well plates inside and outside the humidity chamber. After 96 h the medium in corner, edge and central wells outside the chamber was filled up with distilled / H_2O dest. to equal the volume for the evaporated loss of liquid. Fluorescence intensity of alamarBlue® was measured for **(d)** corner wells (n = 8 per condition), **(e)** edge wells (n = 32 per condition) and **(f)** central wells (n = 60 per condition) of 96 well plates with equal volume inside and outside the chamber. The boxes in the boxplots represent the middle 50% of the data ranging from the lower quartile (Q1) to the upper quartile (Q3). The line within the box indicates the median and the whiskers represent the lower and upper 25% of the data - the minimum and maximum values respectively. P-values are indicated.

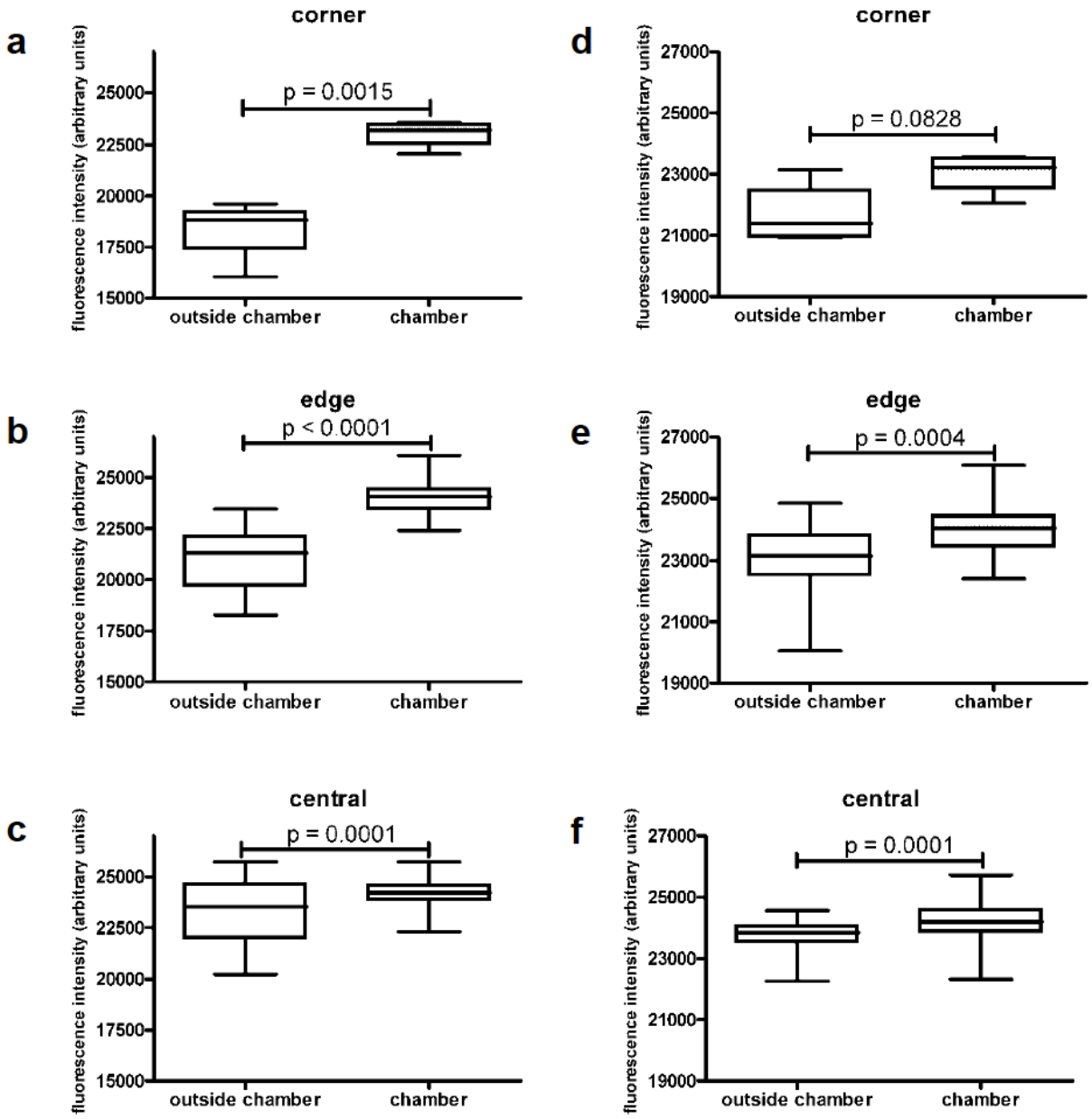

Figure 4. Comparison of intra-plate variation of *alamarBlue® fluorescence intensities dependent on the well position.* AlamarBlue® assay fluorescence intensities of HT-29 spheroids from Figure 3 were compared for intra-plate variation depending on the well position. **(a)** Plate incubated outside the humidity chamber. **(b)** Plate incubated in the humidity chamber; corner wells (n = 8 per condition), edge wells (n = 32 per condition) and central wells (n = 60 per condition) of 96 well plates inside and outside the humidity chamber. The boxes in the boxplots represent the middle 50% of the data ranging from the lower quartile (Q1) to the upper quartile (Q3). The line within the box indicates the median and the whiskers represent the lower and upper 25% of the data - the minimum and maximum values respectively. P-values are indicated.