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Full Length Article

Simple assessment of viability in 2D and 3D cell microarrays using single step digital imaging

Anna A. Popova[a,*], Markus Reischl[b], Daniel Kazenmaier[a], Haijun Cui[a], Timo Amberger[a], Pavel A. Levkin[a,c,*]

[a] Karlsruhe Institute of Technology, Institute of Biological and Chemical Systems – Functional Molecular Systems (IBCS-FMS), Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[b] Karlsruhe Institute of Technology (KIT), Institute for Automation and Applied Informatics, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[c] Karlsruhe Institute of Technology (KIT), Institute of Organic Chemistry, Fritz-Haber Weg 6, 76131 Karlsruhe, Germany

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ABSTRACT

Simple and rapid imaging and analysis of 2D and 3D cell culture compatible with miniaturized arrays of nanoliter droplets are essential for high-throughput screening and personalized medicine applications. In this study, we have developed a simple one-step, cost-effective and sensitive colorimetric method for the analysis of cell viability in 2D and 3D cell cultures on a nanoliter droplet microarray. The method utilizes a flatbed document scanner that detects a color change in response to cell metabolism in nanoliter droplets with high sensitivity in a single step without the need for expensive specialized equipment. This new nanoliter-based method is faster and more sensitive than equivalent methods using multi-well plate assays. The method detects quantifiable signal from as few as 10 cells and requires only 5 min. This is 2.5 to 10-fold more sensitive and 12 times faster than the same assay in multi-well plates. The method is simple, affordable, fast and sensitive. It can be used for various applications including high-throughput cell-based and biochemical screenings.

Introduction

Cell-based high-throughput screening is an essential approach to fundamental scientific research, drug development and personalized medicine. As a state-of-the-art technique, the majority of screenings is performed in multi-well plates in microliter volumes.

To accelerate advances in research and drug discovery, it is important to enable screening of large chemical libraries in cost-effective way. In addition, reducing the amount of cell material required for the experiment might be crucial for some applications, for example for screenings of rare or hard to expand cells, like primary or patient-derived cells. Therefore, it is absolutely necessary to miniaturize screening platforms from microliter to nanoliter volumes and beyond. In recent years, a number of miniaturized platforms have emerged as alternatives to multi-well plates for use in screening applications.

Such platforms are usually not compatible with plate readers or other equipment designed for multi-well plates and alternative analytical techniques are required to facilitate the measurement of different parameters in small culture reservoirs. The platforms based on droplet microfluidic principles, for example, are not compatible with standard spectrophotometers and customized read-out equipment based on absorbance spectroscopy has to be developed for such platforms [1–4]. Digital imaging for estimation of color changes in culture vessels is a cost-effective alternative to complex and expensive spectroscopy [5–7]. Colorimetry is widely used in microfluidic paper-based analytical devices to detect color changes in analytes from different sources, such as food [8,9], environment [8–10], and patient material [11,12]. The change of color is analyzed either by visual detection, or by using digital cameras or scanners [13]. Obtained digital images can be evaluated using computer vision-based analytical procedures and different color spaces (e.g., RGB or grayscale) [13,14].

Colorimetric assays based on changes in the absorbance of a culture media are commonly used in a variety of multi-well cell-based assays, including cell viability and proliferation assays [15,16]. Cell viability is routinely used to determine the cytotoxic effects of tested compounds or materials. Viability assays are based on estimation of cell metabolic activity by measuring biochemical markers such as the presence of the reducing agents NADH and NADPH. These assays involve incubation of viable cells with a specific agent, leading to the a change in absorbance that is proportional to the number of metabolically active cells. The most commonly used colorimetric viability reagents are tetrazolium reduction assays, e.g., WST-8, and the oxidation–reduction indicator, resazurin [15,16].

* Corresponding author
E-mail addresses: anna.popova@kit.edu (A.A. Popova), levkin@kit.edu (P.A. Levkin).

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All these colorimetric assays have been developed for a multi-well plate format and require a microplate spectrophotometer, which is not compatible with nanoliter volumes. In addition, such assays are usually performed on thousands of cells in microliter volumes and, to the best of our knowledge, there are no studies indicating sufficient sensitivity to detect single or very low numbers of cells cultured on miniaturized platforms.

In the current study, we investigated the use of colorimetric viability assays in nanoliter droplets on a droplet microarray (DMA) platform. The DMA is a miniaturized platform for cell-based high-throughput screening based on hydrophilic-superhydrophobic patterning [17–24]. We have demonstrated the possibility to detect cell viability in nanoliter volumes using two commercially available viability reagents, resazurin and [2-(2-methoxy-4-nitrophenyl)−3-(4-nitrophenyl)−5-(2,4-disulfophenyl)−2Htetrazolium, monosodium salt] (WST-8). Our results remonstrate that tested viability assays are more rapid and sensitive in nanoliter volumes compared to multi-well plates. This approach facilitated detection of a quantifiable signal from as few as 10 cells only 5 min after addition of the reagent. Due to the flat nature of the DMA chip, a flatbed document scanner could be used to acquire an image of the whole array and quantify the color changes in the droplets in a single step. This approach represents a cost-effective, rapid and sensitive one-step method to estimat cell viability across the DMA chip. Finally, this method is not only for two-dimensional (2D) monolayer culture, but also for three-dimensional (3D) cell culture models, with the ability to estimate and compare the sizes of single tumor spheroids in nanoliter droplets. Thus, this approach can be adopted to achieve more physiologically relevant screening with a rapid and cost-effective read-out. The application of high-density DMA chips for cell screening in combination with simple methodology for estimation of cell viability developed in this study will be especially important for a potential application for testing patient-derived cells. For example, for testing sensitivity and resistance of cancer biopsy cells to anti-cancer drugs on a chip aiming to identify a suitable therapy for a particular patient. In this case simple, rapid and affordable methods are especially important [25].

Materials and methods

Cell culture

The HeLa CLL2 human cervical adenocarcinoma cell line was purchased from DSMZ GmbH (Braunschweig, Germany), and the HepG2 human liver cancer cell line was kindly provided by Prof. Ute Schepers. HeLa CCL2 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific Inc., Karlsruhe, Germany). Cells were cultured in 10-cm cell culture dishes and split in ratio of 1 to 7 every 2–3 days. Cells were detached from cell culture dishes using trypsin (Gibco, Thermo Fisher Scientific Inc., Karlsruhe, Germany).

2D. cell culture on DMA slides

DMA slides were obtained from Aquarray GmbH (Eggenstein-Leopoldshafen, Germany). In the current study, DMA slides containing 672 square hydrophilic spots in array format of 14 × 48 with hydrophilic spots with a side length of 1 mm were used. Before the cell culture, DMA slides were sterilized in 70% ethanol for 5 min and then dried under sterile bench conditions for 15 min. Cells were trypsinized before seeding according to the standard protocol and counted using automated cell counter Countess II (Life Technologies). Cells were stained with trypan blue and concentration of only live cells was considered. Cell concentration was adjusted accordingly to desired final number of cells in 150 nL volume in full cell culture medium (DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin). Cells were seeded onto DMA slides using the low volume non-contact dispenser I-DOT One (Dispendix GmbH, Stuttgart, Germany) at a volume of 150 nL per spot. During cell dispensing, the humidity was adjusted to 70% by using a humidifier (CleanAir Optima), which is connected through the tubing to an inside chamber of I-DOT One, where the humidity sensor is attached. Cells were dispensed onto one DMA with eight parallel channels under air pressure of 150 mbar/ms; dispensing of one array of 672 spots took 44 s. After dispensing, the DMA slide containing cells was immediately placed inside a 10-cm Petri dish, 2 mL in total of Dulbecco's phosphate-buffered saline (PBS) was added on both sides of the DMA slide and Petri dish was covered with a lid containing a humidity pad soaked with Dulbecco's PBS. The Petri dish was placed in standard cell culture incubator (37 ˚C and 5% CO_2) and the cells were cultured on DMA slides for 24 h with or without compound treatment before staining was performed.

3D. spheroid culture on DMA

HepG2 cells (40 to 300 cells per spot) were seeded onto DMA slides as described in "2D cell culture on DMA slides". Immediately after seeding, the DMA slide containing cells was placed onto a specially designed table in the inverted position within a 10-cm Petri dish [20]. Spheroids were formed in hanging droplets on the DMA slide during the 72 h before staining with WST-8 and resazurin described in "Resazurin and WST-8 viability assay on the DMA".

Resazurin and WST-8 viability assays in 384-well plates

WST-8 and resazurin viability assays were performed in 384-well plates according to the manufacturer's protocol. Cells (from 1 to 3000 cells per well) were seeded manually in 384-well plates in 30 μL of full cell culture media and incubated for 24 h. Viability assays were performed by adding 10 μL of either WST-8 (Kit-8, Dojindo EU GmbH, Munich, Germany) or resazurin (Sigma-Aldrich Chemie, Munich, Germany) to each well. Afterwards, the plates were incubated in cell culture incubator at 37 ˚C and 5% CO_2 between 20 min and 5 h, depending on the experimental setup. For the WST-8 assay, absorbance was measured at 450 nm and for the resazurin assay, absorbance was measured at 570 and 600 nm using SpectraMax iD3 plate reader (Molecular Devices, San Jose, USA). For the analysis, the absorbance of the medium control was subtracted from the measurement of each well. For the resazurin assay, the absorbance at 600 nm was subtracted from the absorbance at 570 nm.

Drug treatment

The following drugs were used in the study: doxorubicin (Alfa Aesar by Thermo Fisher, Kandel, Germany), pazopanib (Cayman Chemical, Ann Arbor, USA), vorinostat (Abcam, Berlin, Germany), nilotinib (Cayman Chemical) and dasatinib (Acros Organics, Thermo Fisher Scientific Inc., Karlsruhe, Germany).

For drug treatment on the DMA platform for each drug 4 mM solutions were prepared in DMSO and then used to prepare 7.5 μM and 750 μM stock solutions in DMSO. Drugs were dispensed directly onto hydrophilic spots of sterile and dry DMA slides using the sciFLEXARRAYER S11 liquid dispenser (Scienion, Berlin, Germany). For final concentrations of 0.05 μM, 0.1 μM, 0.5 μM and 1 μM, 1 nL, 2 nL, 10 nL, and 20 nL of the 7.5 μM stock solution were dispensed, respectively. For final concentrations of 5 μM, 10 μM, 25 μM, and 50 μM, 1 nL, 2 nL, 5 nL, and 10 nL of the 750 μM stock solution were dispensed, respectively. In addition, 10 nL of DMSO was used as a control. After dispensing, DMA slides containing the pre-printed drugs were dried under high vacuum for 2 h and used in cell experiments on the next day. Cells were incubated with drugs for 24 h before performing staining and viabiltiy assay.

For drug treatment in 384-well plates HeLa-CLL2 cells were seeded in 384-well plates in amount of 3000 cells per well in 30 μL of full cell

culture media and incubated overnight. Anti-cancer compounds (doxorubicin, pazopanib,vorinostat and dasatinib) dissolved in DMSO were added to achieve the final concentrations of 0.05 μM, 0.1 μM, 0.5 μM, 1 μM, 5 μM, 10 μM, 25 μM and 50 μM. The final concentration of DMSO was not exceeding 1% from final volume. Cells were incubated with drugs for 24 h. Cell viability was estimated using WST-8 (Kit-8, Dojindo EU GmbH, Munich, Germany) viability assay, which was performed in 384-well plates according to the manufacturer's protocol as it is indicated in "*Resazurin and WST-8 viability assays in 384-well plates*".

Dose–response curves were plotted in OriginPro using "Nonlinear curve fit," category "Growth/Sigmoidal," function "DoseResp," iteration algorithm "Levenberg Marquardt," and multidata fit mode "Independent fit." IC_{50} values were calculated in OriginPro after curve fitting.

Resazurin and WST-8 viability assays on the DMA

Cell staining was performed by dispensing 100 nL of either WST-8 (Kit-8, Dojindo EU GmbH, Munich, Germany) or resazurin (Sigma-Aldrich Chemie, Munich, Germany) directly onto each droplet containing cells using the low volume non-contact dispenser I-DOT One. After addition of the reagent, the DMA slide loaded with cells was returned to the cell culture incubator and incubated between 20 min and 5 h, depending on the experimental setup. After the incubation, the DMA slides were scanned using a scanner CanoScan 8800F (Canon, Inc. Tokyo, Japan) using the following settings: color, 80% of light, 6400 dpi. Scanning of the full slide was completed in 6 min. Three rows from each side were excluded in the image analysis due to evaporation during scanning from all 4 sides of the slide.

Microscopy

For estimation the number of cells per spot, cells were stained with Calcein AM (Thermo Scientific). An aliquot (50 nL) of a solution containing 1.5 μg/mL Calcein AM (Thermo Scientific) in Dulbecco's PBS was dispensed directly onto each droplet using the I-DOT One dispenser. cells were incubated at 37 °C for 15 min before imaging. The whole slide was first imaged under 2 × magnification using a Keyence BZ-9000 microscope (Keyence, Osaka, Japan). Each spot was then imaged under 10 × magnification using an automated screening microscope Olympus IX81 microscope (Olympus, Tokyo, Japan).

To estimate the number of cells per spot after drug treatment, cells were stained with Hoechst 33342 (Thermo Scientific) by dispensing 50 nL of a solution containing 4 μg/mL of Hoechst in Dulbecco's PBS using the I-DOT One dispenser. Subsequently, the cells were incubated for 15 min 37 °C in cell culture incubator before imaging. An image of each spot was obtained under 10 × magnification using an automated screening microscope Olympus IX81 microscope (Olympus).

For the experimetns presented in Fig. 2 cells were stained by dispensing 50 nL of PBS containing 40 μg/mL Hoechst 33342 (Invitrogen by Thermo Fisher, Eugene, USA), 1,6 μg/mL Calcein AM (Invitrogen by Thermo Fisher, Eugene, USA) and 20 μg/mL PI (Invitrogen by Thermo Fisher, Eugene, USA), so the final concentration of Hoechst, CalceinAM and PI was 4 μg/mL, 0.4 μg/mL and 5 μg/mL, respectively. After dispensing the staining solution the cells were incubated for 30 min in cell culture incubator. Cells were imaged with Keyence BZ-X800 (Tokyo, Japan) at 10x and 20x magnification.

Image analysis

For analysis of scanned images of DMA slides stained with WST-8 or resazurin reagents, the user defined the number of rows and columns to be evaluated, as well as the top left corner and bottom right corner and horizontal and vertical displacement of all wells. The central position of all wells was estimated and a square of 41 × 41 pixels located close to the center was extracted. The intensity of the color-channels was calculated and normalized to [0,1] (for resazurin, the R-channel to $R + G$-channel ratio was calculated; for WST-8, the blue channel was inverted). The intensities were averaged within the extracted square to obtain a single intensity value. To validate the robustness of the quantification, histograms of the normalized pixel intensities of the whole droplet and 41 × 41 pixel area in the middle was calculated for 24 droplets of each condition (in total 96 spots): (a) media control resazurin; (b) cells resazurin; (c) media control WST-8; and (d) cells WST-8 (Fig. S6 and S7). Figures S6 and S7 are showing droplets in high resolution with marked area of 41 × 41 pixels. As can be seen in Figures S6 and S7, pixel intensities vary across the droplet due to edge effects. Therefore, we did not use the whole droplet area for the quantification of color change. And to be robust against measurement artefacts, we heuristically chose an area of 41 × 41 pixel, which demonstrated a homogenous brightness distribution.

Microscope images were analysed using free Fiji ImageJ software (https://imagej.net/software/fiji/). First, the image was converted to an 8-bit image before the threshold was adjusted manually. The "Watershed" algorithm was used to separate cells on the images. The "Analyze particle" function was then used to count the number of stained cells per spot. The "Batch macro" function of ImageJ was applied for automatic analysis of the full set of 672 images.

Results

Colorimetric assays on the DMA

In this study, we have investigated two commercially available colorimetric reagents, resazurin and WST-8 for their performance in assays conducted in nanoliter droplets on a DMA chip (Fig. 1a). Resazurin is a dark blue phenoxazine dye that absorbs at 600 nm. It is reduced by aerobic respiration of live cells to resorufin, which is bright pink and absorbs at 570 nm (Fig. 1a, upper panel). WST-8 is a colorless tetrazolium salt that is reduced by living cells to a bright yellow formazan that absorbs at 450 nm (Fig. 1a). We optimized one-step imaging of the array using food color printed on the DMA chip (Fig. S1). We have used a scanner with 6400 dpi resolution and 80% light exposure to obtain high resolution homogeneous color images of the whole array (Fig. S1a; Fig. 1a, images of arrays). For the image analysis and estimation of color changes in the droplets we have developed an automated algorithm (see "Materials and methods" section). A schematic diagram of the workflow of the viability assay is presented in Fig. 1b. In the first step, a viability dye is dispensed into each droplet of the array containing cells treated with a library of interest (which can be drugs, small molecules, siRNA or other factors) using a non-contact low volume dispenser (Fig. 1b, Step 1). Dispensing of the dye (100 nL) across the whole array is completed in approximately 1 min. In the second step, the DMA is incubated in a cell culture incubator at 37 °C for 20 min. In the third step, the DMA is scanned using a scanner, a process that is completed in approximately 6 min at the required resolution. In the fourth step, the array scan is analyzed using an automated algorithm that was developed in house. The analysis of one slide takes approximately 2 min. The whole workflow is completed in approximately 30 min, is not expensive and does not require specialized imaging equipment.

As a first step, we have tested the possibility to detect live cells on the DMA platform with resazurin and WST-8 viability assays. We have cultured HeLa-CLL2 cells on the DMA platform in 150 nL droplets for 24 and 48 h without medium exchange (Fig. 1). Cells were stained with Hoechst, Calcein AM and propidium iodide (PI) followed by microscopy. The viability of cells on the DMA was 91–95% and 77–87% after 24 and 48 h of incubations, respectively (Fig. 2a and b). In parallel we have stained HeLa-CLL cells cultured with the same conditions with resazurin and WST-8 dyes. As shown in Fig. 2c and d, we could detect obvious color change in the droplets containing cells, compared to the medium control (Fig. 2c and d).

To demonstrate the utility of both resazurin and WST-8 assays on the DMA for estimation of cell viability, we have dispensed a gradient

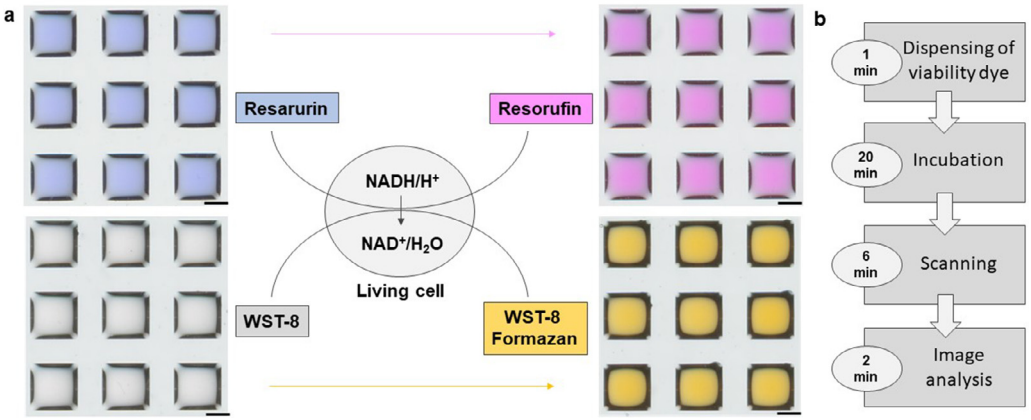


Fig. 1. Schematic representation of the mechanism of resazurin and WST-8 viability assays and workflow of the colorimetric read-out protocol on the DMA platform. (a) Images represent a 3 × 3 array of 250 nL droplets confined in hydrophilic squares with side length of 1 mm and surrounded by a superhydrophobic background. Scale bar: 0.5 mm. (b) The workflow of the colorimetric read-out protocol on the DMA platform.

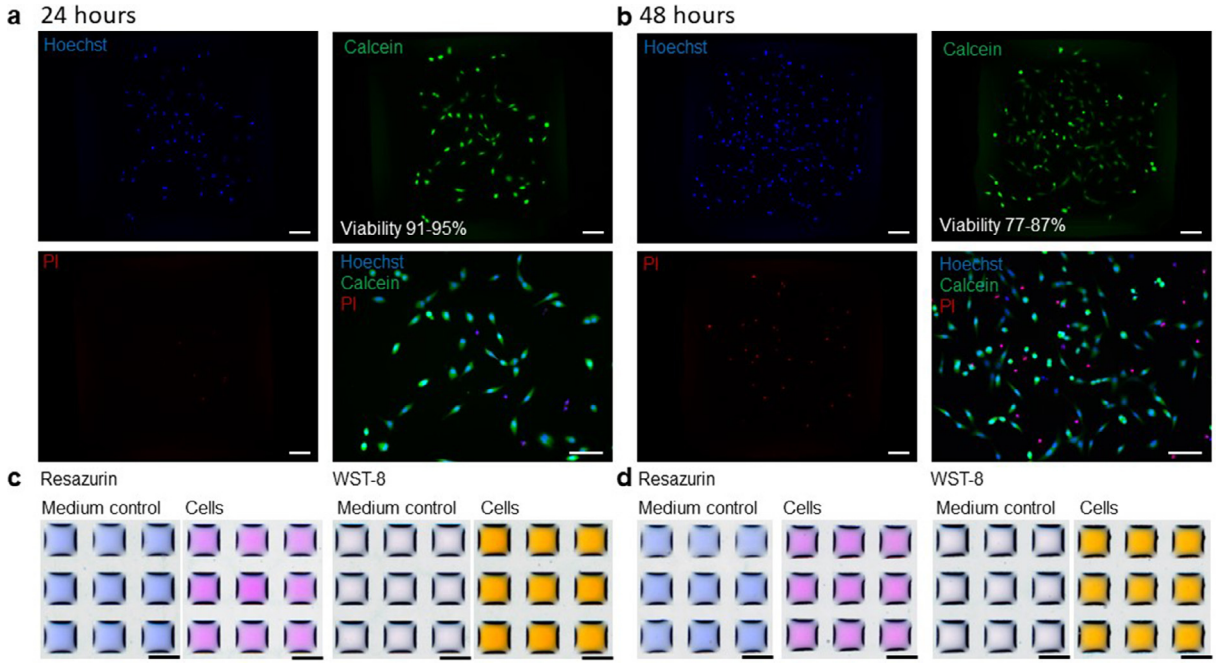


Fig. 2. Culturing and staining of cells on the DMA platform. (a – b) Fluorescent microscope images of HeLa-CCL2 cells cultured on DMA platform for (a) 24 and (b) 48 h and stained with Hoechst, Calcein and PI. Scale bar: 100 μm. (c - d) Detection of live cells on DMA platform with resazurin and WST-8 viabiltiy assay (c) after 24 and (d) 48 h of culture. Scale bar: 1 mm.

of cells (0, 40, 80, 120, 160, 200, 250 and 300 cells per spot) in 150 nL droplets on the DMA as shown in Fig. 3a and incubated in standard cell culture incubator (37 °C and 5% CO_2) overnight. Subsequently, we added 100 nL of either resazurin or WST-8 dye across the whole DMA slide, incubated the cells in a cell culture incubator for 20 min and scanned the whole DMA slide using a scanner (Fig. 3a). In parallel we stained the control DMA slide using Calcein AM followed by quantification of live cells under a microscope (Fig. 3a). Scans were analyzed using an in-house developed algorithm (see Materials and methods) that assigned a number, referred to as “color intensity”, to each spot. As shown in Fig. 3b, a clear gradient of “color intensity” was obtained using both resazurin and WST-8 viability dyes (Fig. 3b, middle and right) that corresponded to a gradient of the number of calcein-positive cells (Fig. 3b, left). It should be noted that variability of color intensity observed for each number of cells per droplet (40 to 300) reflects naturally occurring distribution of number of cells per spot, which can be concluded from similar standard deviations on graphs based on both quantification of live cells and colorimetric assays (Fig. 3b). Therefore, with these results we have demonstrated that both resazurin and WST-8 dyes can be used to detect metabolic activity of as few as 40 cells in 150 nL droplets after incubation for only 20 min. Furthermore, the significant changes in the signal generated by an average of only 40 cells could be detected in a digital image of the cell array obtained with a scanner.

Comparison with 384-well plates

As a next step, we have compared the sensitivity and incubation time for both resazurin and WST-8 viability assays performed on the DMA and in 384-well plate. First, we investigated the time required to obtain a signal from 300 cells using a DMA and in microplates. Using the DMA, the signal from both dyes was already detectable after 5 min of incubation and saturated after 50 min (Fig. 4a and b). In contrast, approximately 1 h (12 × longer) was required to obtain a detectable signal using the 384-well plate, and the signal was not saturated even after 5 h of incubation (Fig. 4a and b). Second, we investigated the minimum number of cells that can be detected on the DMA and 384-well plates using resazurin

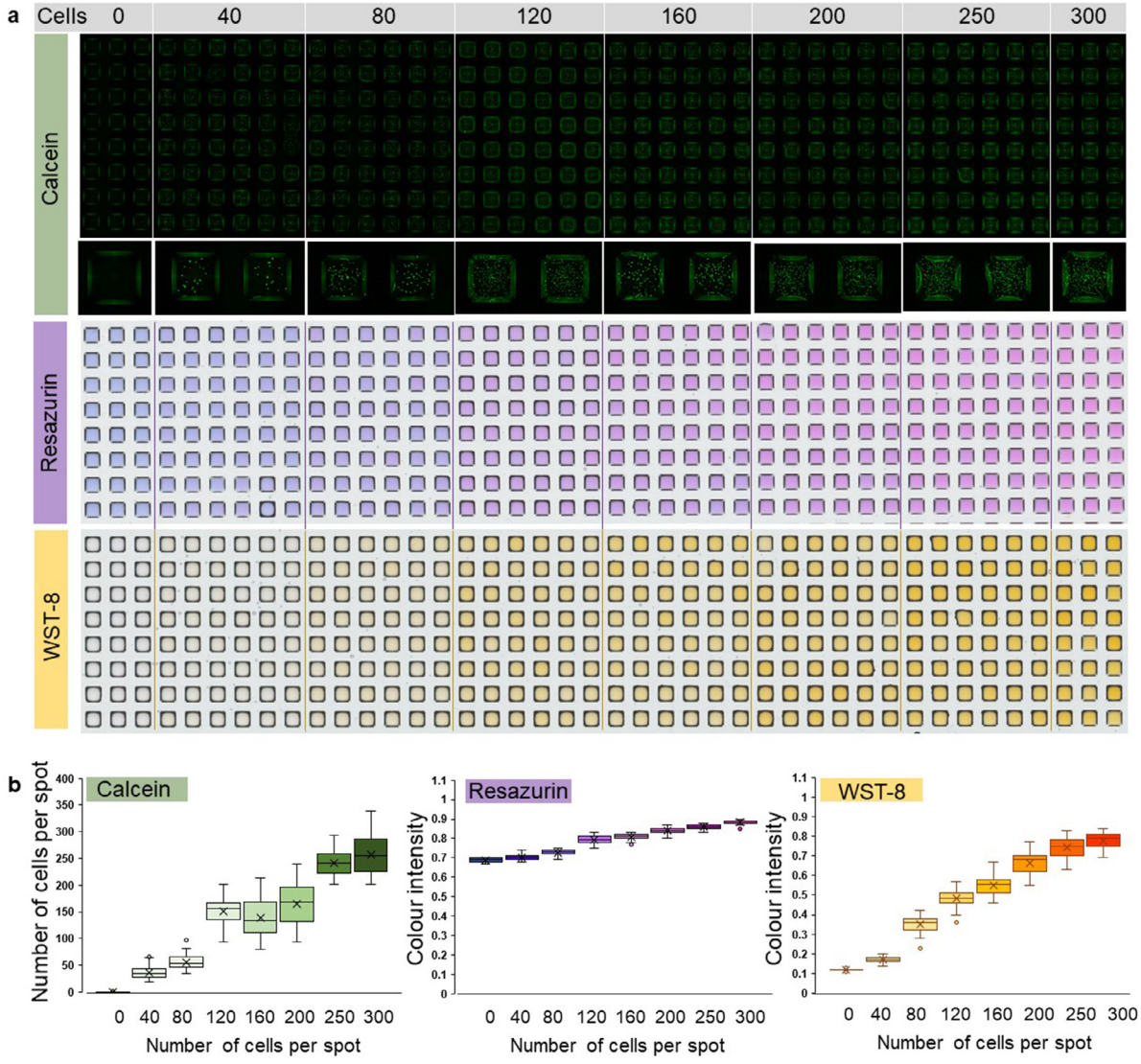


Fig. 3. On-chip colorimetric analysis of cell gradients using resazurin and WST-8 viability assays compared to Calcein AM staining followed by microscopy and cell quantification. (a) Microscope image of a droplet microarray containing a gradient of cells stained with Calcein AM (upper panel); scans of a droplet microarray containing a gradient of cells stained with resazurin (middle panel) and WST-8 (lower panel). (b) Graphs showing the distribution of cell numbers stained with Calcein AM (left panel), and graphs showing the intensity of color measured after staining of cells with resazurin (middle panel) and WST-8 (right panel).

and WST-8 viability assays. We have seeded a gradient of cells ranging from 1 to 300 per spot/well and incubated for 1 h and 5 h on the DMA and 384-well plates, respectively. On the DMA, a signal was detected from both viability dyes using as few as 10 cells per spot, in contrast to 384-well plates, in which 25 and 100 cells were required for detection of signals from WST-8 and resazurin, respectively (Fig. 4c and d). These results indicated that both resazurin and WST-8 viability assays conducted using the DMA are approximately 12 times faster and from 2.5 to 10 times more sensitive than those performed using microplates. Furthermore, using the DMA, both assays can be used to detect as few as 10 cells after only 20 min of incubation time. We speculate that these advantages are due to the markedly reduced (200 × lower) medium-to-cell ratio on the DMA, resulting in more rapid accumulation of metabolites compared to that in assays using multi-well plates.

Drug treatment

To confirm that the established resazurin and WST-8 colorimetric viability assays are suitable for estimation of cell viability after drug treatment, we performed the treatment of HeLa cells with different concentrations of the anti-cancer drug doxorubicin (Fig. 5). We pre-printed different concentrations of doxorubicin onto the hydrophilic spots of the DMA (Fig. 5a). Afterwards we have seeded the HeLa cells onto the pre-printed DMA and incubated for 24 h. To demonstrate that the protocol of drug treatment on the DMA is comparable with commonly used protocols in multi-well plates, we have performed comparable drug treatment in 384 well plates. HeLa-CLL2 cells were plated first, cultured overnight and introduced to 4 different drugs dissolved in DMSO. We have obtained comparable dose-responses and IC_{50} values, which indicates that the procedure of drug treatment on the DMA platform gives relevant results (Fig. S5). In drug treatment protocol on the DMA platform resazurin and WST-8 colorimetric viability assays were performed according to the established protocol (Fig. 5a and S3). We have compared the results obtained with the colorimetric method with those obtained using staining evaluated by microscopy, which is the method usually used in our laboratory for estimation of drug toxicity. As shown in Fig. 5b, the dose-responses of HeLa cells obtained using all three methods were comparable with the half-maximal inhibitory concentration (IC_{50}) values identified as 0.5 ± 0.03, 0.7 ± 0.04 and 0.4 ± 0.2 by microscopy and resazurin and WST-8 viability assays, respectively. With these results we have demonstrated that the colorimetric viability assays established on

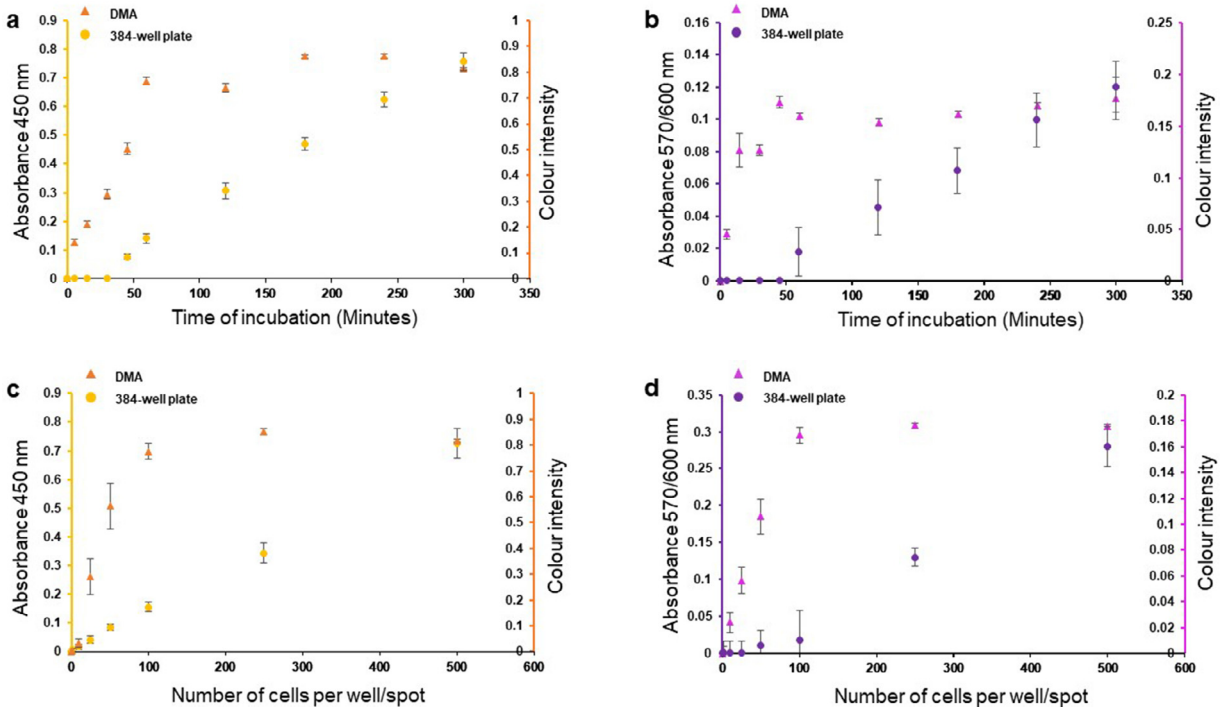


Fig. 4. Comparison of resazurin and WST-8 colorimetric viability assays performed on droplet microarrays (DMAs) and in 384-well plates. (a - b) Graphs showing comparison of signal changes of (a) WST-8 and (b) resazurin viability assays developed using 300 cells per well/spot over a period of 5 h. Error bars on the graph represent a single standard deviation for each data set. For Droplet Microarray and 384-well plates, 48 droplets and 32 wells were used for each measurement, respectively. (c - d) Graphs showing comparison of signal changes of (c) WST-8 and (d) resazurin viability assays developed using the following gradient of cells: 1, 10, 25, 50, 100, 250, 500. Error bars on the graph represent a single standard deviation for each data set. For Droplet Microarray and 384-well plates, 24 droplets and 16 wells were used for each measurement, respectively.

the DMA were suitable for use as a read-out technique in drug toxicity screening applications.

Next, we have evaluated the potential application of the established colorimetric assays as a read-out in drug screenings using experimental setup commonly adopted in real screening experiments. We have printed the drugs in four repeats per concentration and randomly distributed them across the array. We have pre-printed different concentrations of the anti-cancer drugs pazopanib, vorinostat, nilotinib and dasatinib on the DMA (Fig. 6a and S4). Afterward, we have seeded the HeLa cells onto pre-printed arrays and cultured them for 24 h. The dose-responses and IC_{50} values for each drug obtained using the colorimetric assays were compared with those obtained using the microscopy-based read-out (Fig. 6b).

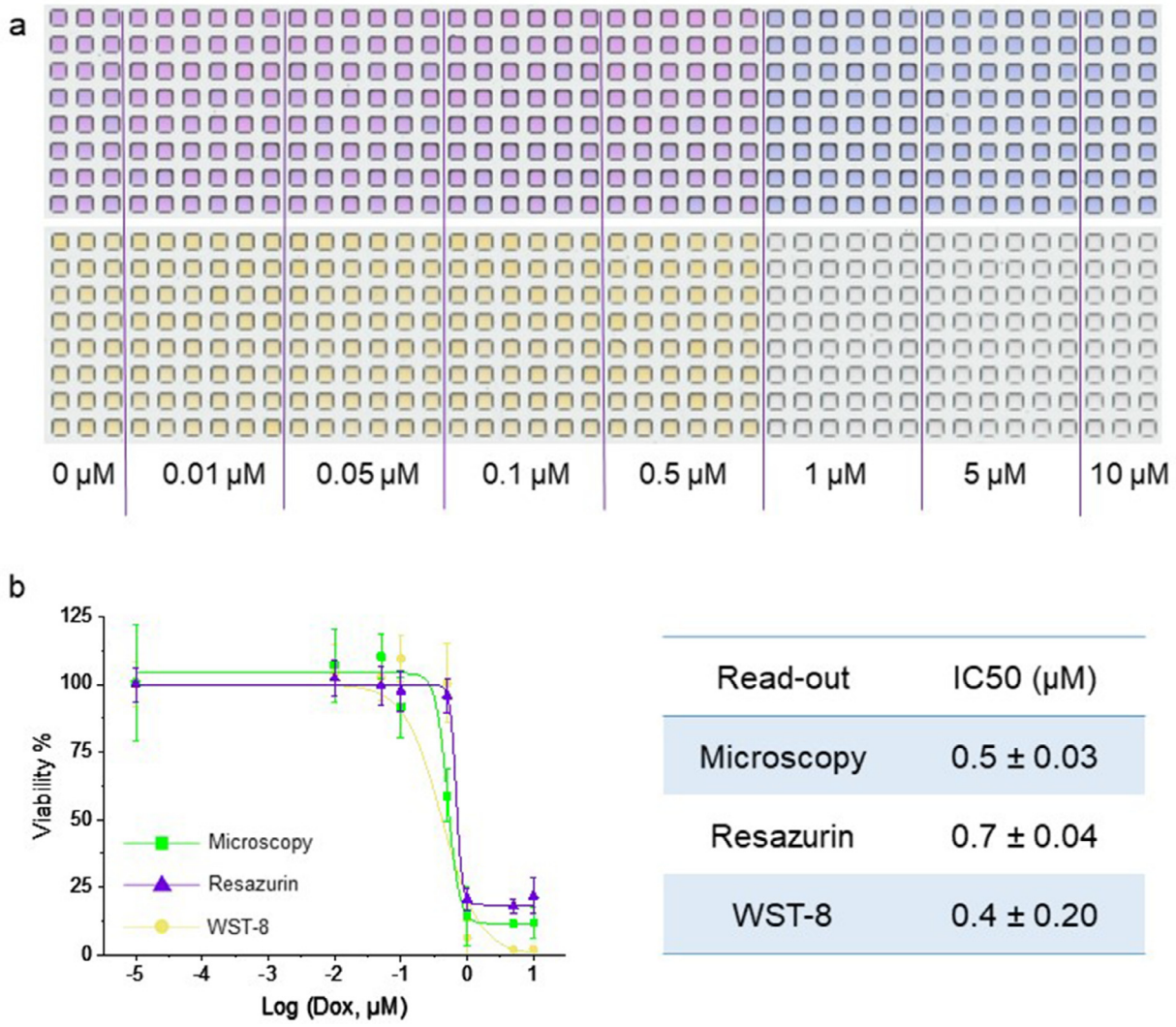
As shown in Fig. 6b, we have obtained comparable dose-response curves and IC_{50} values for all four tested drugs. This confirms that established colorimetric viability assays are suitable for use in high-throughput drug screening applications.

3D. cell culture

In vitro 3D cell culture models more closely resemble the physiological environment in vivo than 2D cell culture systems. To use resazurin and WST-8 viability assays on the DMA for 3D cell culture, we have developed protocols to estimate the sizes of spheroids formed on the DMA chip. HepG2 spheroids were formed on the DMA using a previously developed protocol based on the “hanging droplet” method [20]. To obtain the spheroids of different sizes on one array, we have seeded different numbers of cells ranging from 40 to 300 cell per spot in blocks of 6×14 spots (Fig. 3a). The ability to both precisely control the sizes of spheroids and obtain single-spheroid arrays is highly important for high-throughput screening applications. After cell seeding we have placed the DMA slide containing cells onto a specially designed table in the inverted position within a 10-cm Petri dish in cell culture incubator as described previously [20]. After 3 days in culture, HepG2 spheroids of different diameters formed spontaneously on the DMA (Fig. 7a). We have performed resazurin and WST-8 viability assays on formed spheroids according to established protocols, increasing the incubation time with reagents from 20 to 60 min (Fig. 7). The difference in spheroid diameter was closely reflected in the color change of the resazurin and WST-8 reagents (Fig. 7a and b). With these results we have demonstrated that resazurin and WST-8 viability assays are suitable for use in screening applications based on 3D cell cultures.

Discussion

In the current study, we have evaluated the performance of the commercially available colorimetric viability dyes, resazurin and WST-8, in nanoliter droplets on a miniaturized DMA platform. We have developed a simple, one-step, fast and cost-effective methodology for detection of cell viability on DMA chip. We have used a scanner to acquire a single image of the whole array, and established an algorithm for automatic image analysis to estimate the color changes in each droplet. With our results we have demonstrated the usability of the established protocol for estimation of the viability of 2D cell cultures in drug screening application, as well as for estimation sizes of spheroids. The DMA-based assays can be used to detect signals from nanoliter droplets within 5 min compared with the 1 h required for multi-well plates, thus representing a 12-fold faster assay time. Furthermore, the DMA-based assay facilitated detection of as few as 10 cells, representing a 2.5-fold and 10-fold increase in sensitivity for WST-8 and resazurin, respectively, compared with the multi-well plate based assays. We hypothesize that this effect is due to the 200 times lower medium-to-cell ratio on the DMA in comparison with that for the multi-well plates.



Read-out	IC50 (µM)
Microscopy	0.5 ± 0.03
Resazurin	0.7 ± 0.04
WST-8	0.4 ± 0.20

Fig. 5. Comparison of resazurin and WST-8 viability assays and microscopy-based read-out for estimation of drug dose-response. (a) Scans of droplet microarrays (DMAs) containing HeLa cells treated with different concentrations of doxorubicin and stained with resazurin and WST-8. (b) Graph showing a comparison of the dose-response of HeLa cells to doxorubicin obtained using three different read-out methods: microscopy (estimation of cell viability by counting live and dead cells after live/dead staining), resazurin and WST-8 staining (left). A table summarizing the IC_{50} values of doxorubicin obtained using the three read-out methods (right). Dose–response curves were plotted in OriginPro using "Nonlinear curve fit," category "Growth/Sigmoidal," function "DoseResp," iteration algorithm "Levenberg Marquardt," and multidata fit mode "Independent fit." IC_{50} values were calculated in OriginPro after curve fitting.

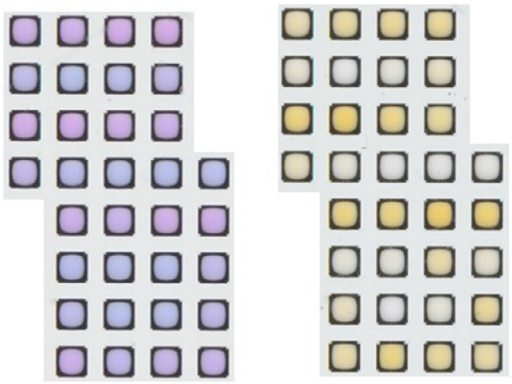
Colorimetric methods of detection are widely used for identification and quantification of molecules in chemical and biological assays. They are less sensitive than fluorescence detection techniques, but have a number of advantages [1,13,26]. Non-fluorescent analytes are characterized by high photostability and relaxation kinetics; therefore, their detection is less prone to the photobleaching, quenching and saturation effects observed in fluorescence-based detection methods. Moreover, fluorescence-based methods require a strongly fluorescent fluorophore with the capacity for rapid relaxation to the ground state [1,27]. As another advantage, colorimetric methods offer the possibility for optical detection of color changes, which can be achieved using non-specialized and inexpensive equipment, such as scanners or phone cameras [13]. This approach is widely used in point-of-care devices, such as microfluidic paper-based analytical devices (mPADs), due to the simplicity, low cost and absence of a requirement for specialized equipment.

Typically, cell-based high-throughput screening techniques rely on complex and expensive equipment for the read-out protocols and optical detection methods based on digital imaging are not applied in such assays. We have demonstrated here that it is possible with the Droplet Microarray platform. The increased sensitivity and flat format of DMA make it possible to apply simple and cost-effective digital imaging for the read-out of complex cell-based assays on the DMA chips.

Rapid and simple optical detection methods can be beneficial for applications like personalized screenings of patient-derived cells. For example, in vitro testing patient cancer cells for their sensitivity to anticancer therapy drugs, which is novel and developing concept in precision oncology [25]. In addition, the methodology developed in this study can be applied for cell-based high-throughput screening in the fields of fundamental research and drug discovery, where large numbers of factors and compounds are tested in parallel and rapid, one-step read-out protocols providing a "yes/no" answer are required.

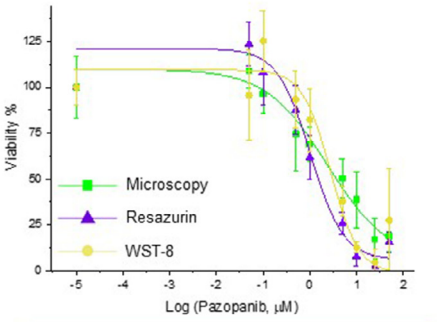
In this study, we have developed a simple, one-step, cost-effective and sensitive cell viability assay on nanoliter droplet microarrays using a scanner followed by automated image analysis. We have presented the method for estimation of viability of cells, cultured in 2D monolayer, as well as for quantification of sizes of single 3D cell spheroids in nanoliter droplets. We have demonstrated the utility of this protocol in a drug screening application. The developed rapid, simple, sensitive

a



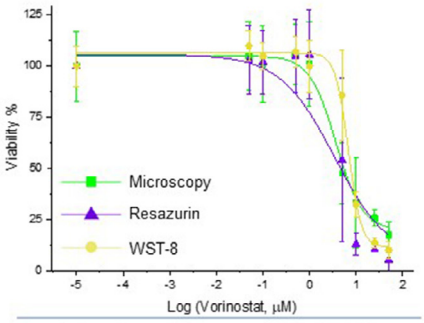
1	0.5	0.1	0.05	
50	25	10	5	
0.05	0.1	0.5	1	
5	50	50	25	10
	1	0.5	0.1	0.05
	10	25	5	50
	5	25	10	1
	0.05	0.1	0.5	0

b



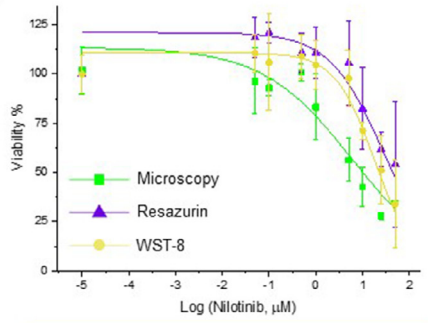
Read-out	IC50 (µM)
Microscopy	2.4 ± 1.3
Resazurin	1.0 ± 0.3
WST-8	2.8 ± 0.8

c



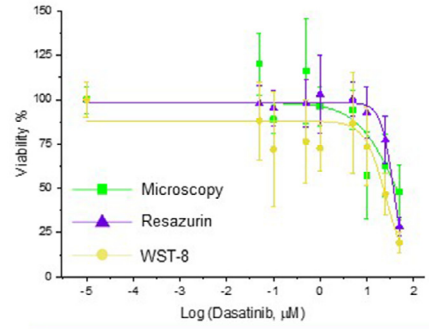
Read-out	IC50 (µM)
Microscopy	3.7 ± 0.8
Resazurin	3.5 ± 0.3
WST-8	6.8 ± 0.5

d



Read-out	IC50 (µM)
Microscopy	10.6 ± 4.2
Resazurin	27.7 ± 4.0
WST-8	18.7 ± 1.7

e



Read-out	IC50 (µM)
Microscopy	44.5 ± 19.8
Resazurin	37.5 ± 1.0
WST-8	30.1 ± 2.7

Fig. 6. Comparison of the performance of resazurin and WST-8 viability assays with a microscopy-based read-out (estimation of cell viability by counting live and dead cells after live/dead staining) in randomized drug treatment on droplet microarrays. (a) Scans of droplet microarrays (DMAs) containing HeLa cells treated with different concentrations of pazopanib and located in random locations stained with resazurin and WST-8 (left). Table showing concentrations of pazopanib (µM_ in random locations corresponding to the scans on the left (right). (b - e) Graphs showing dose-response of HeLa cells to (b) pazopanib, (c) vorinostat, (d) nilotinib and (e) dasatinib and tables summarizing IC_{50} values of drugs obtained using the three read-out methods. Dose–response curves were plotted in OriginPro using "Nonlinear curve fit," category "Growth/Sigmoidal," function "DoseResp," iteration algorithm "Levenberg Marquardt," and multidata fit mode "Independent fit." IC_{50} values were calculated in OriginPro after curve fitting.

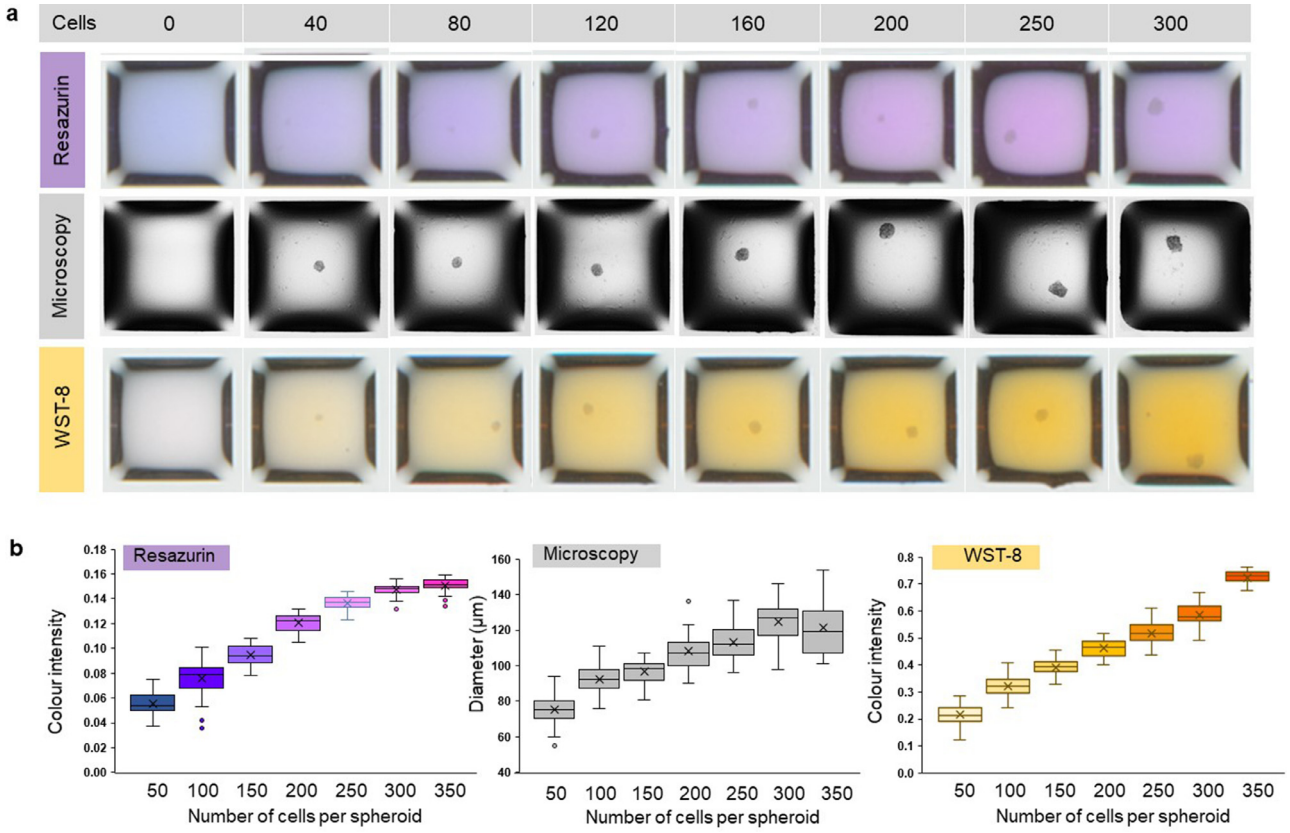


Fig. 7. On-chip colorimetric analysis of spheroids using resazurin and WST-8 viability assays. (a) Scans and microscope images of droplets confining spheroids formed using different cell numbers. Different numbers of HepG2 cells were seeded in 200 nL per spot and cultured in an inverted "hanging droplet" position for 72 h. Appropriate volumes of resazurin (upper panel) and WST-8 (lower panel) viability assay reagents were added to each spot and the intensity of the color was measured after incubation at 37 °C for 40 min. (b) Graphs showing the size distribution of the formed spheroids (middle panel), and analysis of the color intensity for resazurin (left panel) and WST-8 (right panel) viability assays. Error bars on the graph represent a single standard deviation for each data set. A total of 24 droplets were used for each measurement.

and affordable method for the quantification of cell viability in a high-throughput way using a scanner provides great potential for use in applications for testing rare cells (e.g., patient derived biopsy cells), as well as for high-throughput screening in the fields of fundamental research and drug development.

Declaration of Competing Interests

The authors declare the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: in addition to being employed by the Karlsruhe Institute of Technology, A.A.P. and P.A.L. are (since March 2018) shareholders of Aquarray GmbH. M.R., D.K., H.C. and T.A. declare that there is no conflict of interest regarding the publication of this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.slast.2021.10.017.

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