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Microwell arrays reveal cellular heterogeneity during the clonal expansion of transformed human cells

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Abstract

We developed micromolded microwell arrays to study the proliferation and senescence of single cells. Microwell arrays were designed to be compatible with conventional cell culture protocols to simplify cell loading, cell culture, and imaging. We demonstrated the utility of these arrays by measuring the proliferation and senescence of isogenic cells which expressed or had been depleted of the human Werner syndrome protein. Our results allowed us to reveal cell-to-cell heterogeneity in proliferation in WRN⁺ and WRN-depleted fibroblasts during clonal growth.

INNOVATION

Current micro-fabricated devices for studying single cell clonal expansion require microfluidic specialists to operate. Further, the enclosed microfabricated chambers for cell culture have the need of medium perfusion resulting in the difficulty to compare the results with the conventional study. Therefore, we developed a simple polydimethylsiloxane (PDMS)-based microwell device which is bonded onto a cell culture petri dish to form an array of open microwells for the ease of tracking single cells. The growth of the cells is under the conventional cell culture technique which can be monitored using standard imaging equipment and culture protocols. Our PDMS-based microwell culture platform is simpler to implement than other parallel culture or automated single-cell tracking protocols. Consequently, our microwell arrays are easy to use, inexpensive and can be adapted easily by research groups to rapidly track large number of single cells, acquire images, and analyze data.

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AUTHOR CONTRIBUTIONS

The design of these experiments were developed by T.C., A.F. and R.M. Microfabrication and cell culture experiments were conducted by T.C. A.R. and W.T. Modeling of experimental data was performed by M.E. and W.K. All of the authors contributed to writing the final manuscript.

COMPETING INTERESTS STATEMENT

All authors have no financial disclosure and no competing interests to declare.

NARRATIVE

Cell proliferation and senescence are key determinants of the growth of cell populations¹. Cell-to-cell heterogeneity in proliferation, growth potential, morphology, and response to therapy have long been noted in both normal and tumor cell populations². An important challenge in better understanding tumor cell populations and their response to therapy is to improve our understanding of cellular heterogeneity. An important experimental approach to the analysis of cellular heterogeneity has been the use of single cell-derived cultures, often referred to as clonal cultures, to reveal and quantify differences in cellular behavior. One widely used version of this approach in both toxicology and cancer cell biology is to determine the colony-forming efficiency (CFE) of individual cells after treatment with a drug or therapeutic agent³. This assay has been further elaborated with the development of specialized cell culture plates to array and then follow the growth of large numbers of single cells over time. A prominent early example of this approach was the development of micro droplet culture plates for HLA typing of human lymphocytes by Terasaki and colleagues⁴ which were subsequently used to follow the proliferation of individual lymphocytes. Widely used contemporary examples of this approach to cell culture miniaturization and the analysis of single or small numbers of cells can be seen in the many assays and protocols adapted to be performed in 96-well plates⁵. Other similar cell culture systems based on a microfabricated array have been established for trapping and analyzing single cells^{6,7}. However, the PDMS culture substrate with small cell-trapping wells could not be used for longer term clonal expansion study^{6,7}.

We have extended this approach by developing PDMS microwell arrays which have been designed to simplify the seeding of single cells in microwells. Unlike other microwell-based systems⁶⁻⁸, our microwell array utilizes a conventional plastic substrate for cell culture. The arrays are chemically bonded onto dry cell culture dishes before addition of any solutions. The growth and senescence of many parallel cultures initiated from single cells can then be monitored using conventional cell culture and imaging equipment and protocols. The cell culture substrate and both the ease and versatility of use of this system simplify a wide range of experimental protocols. Our platform should as a result be familiar to a large number of scientists, easy to use for a variety of purposes, and allow the generation of data directly comparable to the vast majority of prior single-cell and colony-based growth assays on cell culture surfaces.

In order to demonstrate the ease of use of microwell arrays, we fabricated and used 512 well and 900 well microarrays to characterize the proliferation and senescence of isogenic human cells which either expressed or had been depleted of the Werner syndrome protein WRN. This human RECQ helicase plays important roles in DNA replication, recombination and genome stability assurance⁹. Heritable inactivating mutations in the *WRN* gene cause Werner syndrome, a cancer predisposition syndrome with features of premature aging¹⁰. The loss of WRN function in somatic cells leads to marked suppression of cell proliferation, an increase of cellular senescence and selective sensitivity to cell killing by several important classes of cancer chemotherapeutic agents^{10,11}.

Open chamber PDMS-based microwell arrays

PDMS microwells were generated using an exclusion molding protocol¹². The resulting array, once released from the master and treated with oxygen plasma, can then be bound to cell culture grade polystyrene dishes coated with aminopropyltriethoxysilane (APTES)¹³ to create an irreversible, leak-proof bond between the PDMS microwell array and a polystyrene cell culture substrate. This molding and assembly process is shown for a 900-microwell array in Fig. 1.

In order to demonstrate the reliable exclusion molding technique to generate open microwells, we assembled a montage of a large image array as shown in Fig. 2. The complete 512 PDMS microwell array with cell seeding provides the visual appearance across the device with individual open microwells focused on the cell culture surface.

Poisson distribution of cells in microwells after cell seeding

The design of microwell arrays can be easily and reliably seeded with a desired average number of starting cells. The actual number of cells that are seeded in each individual microwell cannot be controlled, but the distribution (known as Poisson distribution) is very well understood and allows us to predict the proportion of wells seeded with different numbers of starting cells. We then empirically determined the optimal cell concentration and culture medium volume needed to obtain the highest percentage of microwells containing single cells. For 900 well arrays (Fig. 1e), we found that flooding microwell arrays in 10 cm dishes with 20 mL of culture medium containing 1000 cells/mL resulted in the reliable seeding of ~1/3 of the microwells (32%) with single cells. The fraction of wells containing 0 cells served to confirm our predicted distribution, and was also useful for monitoring potential cross contamination between wells or from the PDMS surface (Fig. 1c). Neither type of well contamination was common: 3% of wells initially observed to contain 0 cells was found to contain 1 or more cells at the end of growth experiments of up to 7 days. Using the Poisson distribution to predict cell seeding in the microwells also allowed an easy way to determine whether proliferative behavior is strongly influenced by the starting number of cells in a microwell. As shown in Fig. 3, the procedure was done by identifying wells seeded with 0, 1, 2 or 3 cells at immediately after seeding, and then following all wells over 7 days to determine the proliferative potential of wells as a function of initial cell number. As a result, the probability of proliferation increased as more cells were seeded into microwells, but that this increase was not proportional.

Cell proliferation during clonal growth in microwell cultures

We performed pilot experiments to follow cell proliferation in microwell cultures. These experiments used GM639 SV40-transformed human fibroblasts^{10,14} and a 512 well round microwell array in which we followed the number of cells per well over a 7 day growth period. Daily imaging of microwells seeded with single cells was used to systemically track cell number/well as a function of time. This approach revealed remarkable heterogeneity in the proliferative behavior of single cells. Four different clonal proliferation patterns could be identified: Exponential proliferation (38% of wells); slow or variable proliferation (30%); non-dividing viable cells (7%); and cells which were lost from the wells and were presumed to have died (25%). The combined percentage of wells showing proliferation (68%) closely

approximates the colony forming efficiency of GM639 cells as determined by conventional dilute plating colony forming efficiency (CFE) assay^{9,11,15}.

We next compared the proliferative behavior of isogenic cultures of the same GM639 SV40-transformed human fibroblasts which had been depleted or mock-depleted of the WRN RECQ helicase protein. We showed in previous work that cells depleted of WRN displayed the same defects in cell proliferation, senescence and DNA damage sensitivity as had been previously observed in primary and SV40-transformed fibroblast cultures derived from Werner syndrome patients^{9,11,15}. WRN-depleted and control (mock-depleted) GM639 SV40 fibroblast cells were made by transducing GM639 cells with a lentiviral vector expressing either a shRNA that targeted the *WRN* gene and depleted the corresponding WRN protein^{9,11,15}, or a scrambled shRNA with no known target sequence in the human genome. We had shown in previous experiments that changes in cellular phenotype observed in WRN-depleted cells reflected WRN depletion, as opposed to shRNA-specific off-target effects, lentiviral infection or engagement of the RNAi machinery^{11,16}.

Figure 4 shows individual well and mean growth curves for 139 WRN+ (control/mock-depleted) and 130 WRN-depleted single GM639 cells (189 WRN+ and 195 WRN- single cells from the original seeding) grown in wells in a 512 microwell array. The extent of WRN protein depletion in this experiment was 94%, as verified by Western blot analysis prior to the start of the experiment. Previous work has shown that this level of depletion has reliably revealed WRN-dependent defects in GM639 cells^{11,15}. Growth curves were plotted for wells seeded with 1 WRN+ or WRN-depleted cell/well that had at least 1 cell on day 3 in order to reveal WRN-dependent differences in the proliferative behavior of otherwise isogenic starting cells. This experiment revealed a remarkably wide range of growth trajectories for individual cells which was not apparent from the mean growth curves.

Measuring cell viability and senescence in microwell clonal growth

Cell proliferation is only one of the variables determining the rate of growth of cell populations over time. Other important determinants of total cell number during clonal growth are cell viability and senescence. We were able to identify both cell viability and cellular senescence in microwell array cultures by taking advantage of sequential imaging data and cell staining at the end of a growth period.

GM639 cells that die during clonal growth develop a rounded morphology and detach from the microwell, and are easy to identify by a combination of phase contrast imaging and live-dead staining (LIVE/DEAD cell viability assay, Invitrogen, Carlsbad, CA). In this assay dead cells that lose plasma membrane integrity are stained with red-fluorescent ethidium homodimer-1, whereas live cells are stained with green-fluorescent calcein-AM. This combination of staining and imaging allowed us to identify both viable and dead cells in microwell cultures (Fig. 5).

Senescent cells also fail to proliferate, but remain viable and metabolically active despite the absence of cell division¹. Potentially senescent fibroblast cells are easy to identify on the basis of their distinctive so-called 'fried-egg' morphology alone as shown in Fig. 6, and can be further confirmed by staining for senescence-associated beta-galactosidase (SA- β gal)

activity¹⁴. Identifying both dead and senescent cells can be addressed by LIVE/DEAD assay staining followed by fixation with 4% para-formaldehyde prior to SA- β gal staining (Fig. 6).

DISCUSSION

We developed a microwell array that could be used with existing cell culture, imaging equipment, and protocols to quantify cell behavior across hundreds of individual cells during clonal expansion in culture. The physical separation created from microwells prevents clonal populations from overlapping during a long growth period. Microwell arrays can be fabricated in a wide range of formats, and up to 3000 microwells can be easily handled in a single 10 cm culture dish. These microwell arrays can be modified easily to generate different well geometries, and can be bonded or conformed on pre-coated or nano-patterned substrates to allow the growth and analysis of many different cell populations of high biological or medical interest, e.g., ES or iPS cells, and their lineage-specific, differentiated progeny.

Our use of a cell seeding strategy that takes advantage of Poisson statistics provides a very simple and highly reproducible way to seed a predefined fraction of wells with single (or other defined numbers of) cells, while minimizing cell manipulation and the potential for damage. The maximum percentage of seeded wells is defined by Poisson statistics on any given seeding, thus it is limited and not possible to improve the number of wells seeded with single cells using a single random seeding. However, due to the simplicity of operation of our microwell platform larger numbers of wells seeded with any predefined cell number can be set up rapidly when desired by simply adding more plates. A number of cell trap approaches¹⁸ or robotics-enabled well seeding would also bypass the Poisson limitation, at the cost of further device development or expense in equipment.

We chose a daily (24 hours) imaging interval in our experiments, as this represents a common division time for many human cells in culture¹⁹. Depending on the purpose of the experiments, imaging on shorter or longer timelines can be readily achieved using our PDMS microwell arrays. More data points collected from single cells within the first 24 hours will allow us to study cell division with minimum influence from cells conditioning the medium or from cell-cell contact. In our current setting, the experiment can be performed at very short time intervals (minutes to 1 hour) by using an on-stage incubator to ensure consistent culture conditions. Imaging on longer timelines may be important for very slowly dividing cells, or where cell differentiation in conjunction with proliferation is to be analyzed (e.g., in the proliferation and differentiation of neural precursor cells derived from ES or iPS cells). Our microwell array does not incorporate fluidic inlets so it cannot presently be used for drug screening; it only identifies culture conditions that either facilitate or inhibit single-cell clonal expansion. A better choice for the screening of large numbers of compounds would be a conventional high throughput, robotics-enabled drug/small molecule/RNAi screening platform which is optimized for throughput.

In addition to the demonstration of our microwell platform, we used common statistical methods to compare the contribution of cell proliferation and senescence of cell population growth in microwell cultures. We applied our existing data ($n = 1$) of isogenic WRN⁺ and

WRN-depleted GM639 fibroblasts to demonstrate the statistical analysis for future studies. The detailed analysis is described in supplementary material including Supplementary Fig. 1–4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fabrication of 900-well PDMS microwell arrays. **(a)** Photolithography was used to fabricate masters for the microwell array the generated by PDMS exclusion molding. **(b)** Assembly of a PDMS microwell array to a polystyrene dish. The PDMS microwell array is surface-treated and bonded to a conventional 10 cm polystyrene cell culture dish. Microwell array membranes can also be bonded or conformed onto other cell culture-compatible pre-coated or pre-patterned substrates. **(c)** Cell seeding protocol. Arrays are surface-treated prior to cell seeding by UV sterilization, oxygen plasma treatment to generate a hydrophilic surface, and finally poly-D-lysine surface coating. Cell seeding is done in cell culture medium (20 mL) dispensed over the microwell array in culture dishes under unit gravity. After 2 hours, excess cells can then be removed prior to identifying the number of cells contained in each well at t_0 . **(d–e)** Example of PDMS microwell membrane prior to **(d)** and after **(e)** bonding to a conventional 10 cm polystyrene cell culture dish to form the completed microwell array.

Figure 2.
A montage of 512 microwell image array. The images were taken 2 hours after cell seeding.

Figure 3.

Probability of cell proliferation as a function of starting number of cells/well and WRN protein expression. The proliferative potential in wells seeded with 1, 2 or 3 cells increases, as does the probability of the cell proliferation as a function of starting cell number.

Figure 4.

Proliferation of single human cells in microwell arrays. **(a,b)** Number of cells/well in wells seeded with single WRN+ or WRN-depleted cells after a 7 day growth period shown as a bar graph **(a)** or growth curve **(b)**. Wells were traced every 24 hours by acquiring focused images, and the cell number in each well counted. Black curves in **(b)** represent the mean increase in cell number over time across all single cell wells. The red curves depict the proliferative behavior of the wells shown in **(c)**. **(c)** Phase contrast images of the proliferation of a single cell in a round 500 μm diameter, 400 μm high microwell over 7 days. These wells were chosen because their proliferative behavior (red curves in **(b)**) follows most closely the average behavior (black curves in **(b)**).

Figure 5.

A subset of a large microwell array with live/dead stain. After a 7 day growth period, live/dead cell viability assay (live: green; dead: red) was used to label cells before the images were taken. Each image is an overlay of green and red channel with bright field channel.

Figure 6. Microwell imaging and cell staining to distinguish among live, dead and senescent cells. **(a)** Phase contrast microscopy reveals potential senescent cells which have a ‘fried egg’ morphology with greatly extended cytoplasmic projections and a rounded up, centrally-placed nucleus (arrows). Senescent cells may also have more than one nucleus, possibly the result of cytokinesis failure¹⁷. **(b)** A single viable cell which excludes ethidium homodimer-1 but is stained by calcein-AM and senescence-associated SA-βgal staining.