3D Cell Culture in Anchored Hydrogel Droplets for High Throughput Screening

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ABSTRACT

Studying the response of cells cultured in a physiologically relevant 3D environment has recently shown promise for increasing drug-discovery success rates, compared to cells cultured on standard 2D microplates. However, tools for culturing cells in 3D are challenging to use and difficult to automate. To address this need, ARLD developed two types of plates comprised of surface engineered structures designed to anchor cell-containing hydrogel drops. The Nano-Droplet Array Plate (nDAP) is designed for high density arrays of nanoliter droplets and the Anchored Immersion Microplate (AIM) lids are built for microliter droplets and are fully compatible with standard 96/384 microplates. Hydrogel droplets containing cells are easily dispensed and cured onto the nDAP and AIM surface structures and cultured in growth medium without adhesion loss. Results from the long term culturing of cells (> 30 days) as well as the diffusion of dyes and peptides through the hydrogel, will be presented.

Keywords: dispensing, AIM lid, nDAP, alginate, collagen

1 INTRODUCTION

University researchers as well as large pharmaceutical companies need improved technologies for discovering new drugs. Current drug-discovery approaches are highly inefficient, as <10% of new drugs entering the pipeline receive FDA approval[1]. The tools used today may explain this low success rate as researchers typically study the average response of a large number of cells cultured in standard microplates; a non-natural, 2D environment. Studying the response of cell spheroids and micro-tissues cultured in a more physiologically relevant 3D environment has recently shown promise for increasing the drug-discovery success rate[2]. Similarly, isolated lymphocytes, cultured in 3D, are an important source of new biological drugs.

ARLD has developed two systems for anchoring hydrogel droplets. The nano-Droplet Array Plates (nDAPs) are designed for droplets ranging from 300 picoliters to 3 microliters. High density arrays, exceeding 500 droplets/cm^2, have been prepared on glass microscope slides as shown in Figure 1.

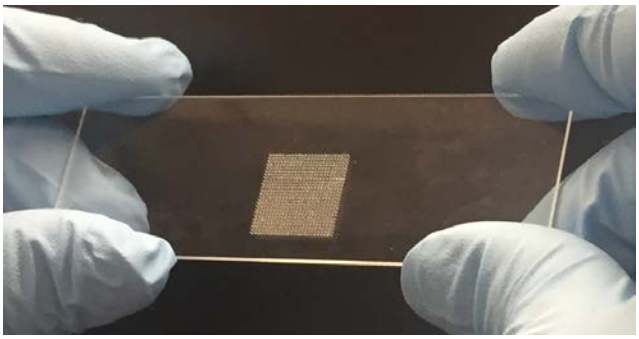

Figure 1. A 25 x 25 nDAP array formed on a microscope slide designed to hold 625 droplets, with a volume of 8.5 nano-liters.

Anchored Immersion Microplate (AIM) lids, for anchoring droplets ranging from 1.5 to 15 microliters, are compatible with standard 96 and 384 well microplates as shown in Figure 2. These systems provide researchers with low-cost and highly reliable tools that will transform the predicative accuracy of new drug discovery research by providing well-controlled 3D hydrogel environments in which cell spheroids, micro-tissues or single cells, can be studied using existing high content/high throughput infrastructure.

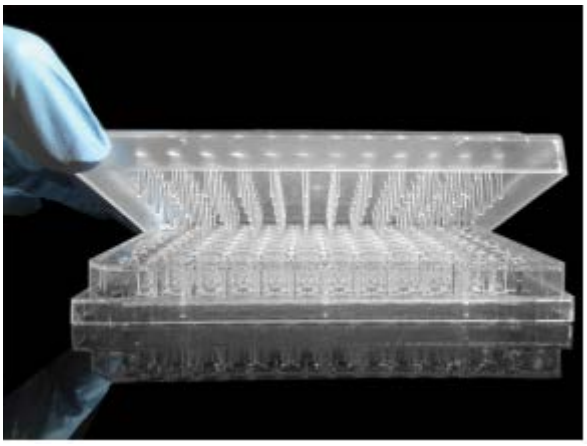

Figure 2. An AIM lid poised above a standard 96 well microplate. Hydrogel droplets containing cells are anchored on each structure that can be immersed and cultured in isolated wells. AIM lids for 384 well microplates are also available.

Both nDAPs and AIM lids employ the same core surface engineering technology. Liquids of any surface tension and viscosity can be handled, enabling the precise dispensing of pico, nano or microliter volumes while remaining highly tolerant of mechanical misalignment, thereby dramatically reducing the cost of the dispensing system. Both nDAPs and AIM lids are compatible with all types of hydrogels including alginate, collagen and matrigel. The hydrogel cross-link density can be adjusted enabling researchers to control the precise environment in which cells are cultured as well as diffusion rates for molecules of interest, ranging from small molecules to peptides to large proteins. Cells dispersed in the hydrogel remain firmly attached to the nDAP and AIM lid surface structures, withstanding multiple manipulations including medium exchange, staining, exposure to drugs and various washing steps.

An overview of the nDAP and AIM lid systems will be presented, including dispense volume CV as a function of surface and process variables. The long-term culturing (≥ 30 days) of several cell types, including cancer cells, will be discussed and compared to conventional 2D cell culture results. Lastly, the imaging of cells in 3D gels with high resolution microscopy (63x oil immersion) using fluorescently tagged DNA stains and cell penetrating peptides will be presented.

2 nDAP: NANO-DROPLET ARRAY PLATES

The nDAP system is comprised four components:

1. The nDAP Substrate is composed of an array of posts on which individual drops are anchored. nDAP substrates are available that hold droplets ranging in size from 300 pL to 3 µL. Densities as high as 500 droplets/cm^2, are formed for handling nanoliter droplets, larger volume droplets are fabricated on coarser pitches to insure adequate separation between droplets. Fluids of any viscosity and surface tension ranging from silicone oil to viscous hydrogel solutions can be dispensed with high precision.

2. The Source Well is used to support a droplet on which the source solution is placed. The Source Well is especially useful when only a limited sample volume is available. Source volumes as small as 10 µL can be used to generate nanoliter droplets. Due to the superhydrophobic nature of the surface, unused source solution can be recovered leaving dead volumes of < 1 µL. When larger volumes of source solution are available, conventional microwell plates can be used as source wells

3. The DropRbot is an automated transfer robot that uses a dispensing tip to transfer solutions from the source well to the nDAP posts as shown in Figures 3 and 4. Because the diameter of the nDAP post determines the volume of solution dispensed, low cost robotics can be used to achieve dispense precision of <2% for droplets that measure between 0.3 to 3000 µL. ARLD's proprietary software coupled with the DropRbot's imaging system, enables automated alignment and insures accurate dispensing.

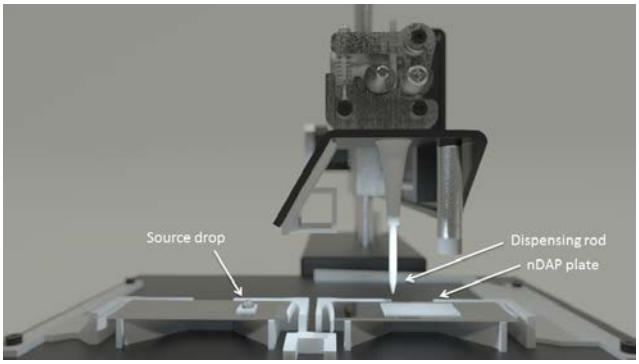

Figure 3. DropRbot automated dispensing system showing source well, nDAP plate and dispensing rod.

The DropRbot is housed within a humidity controlled chamber to prevent droplet evaporation when dispensing nanoliter and picoliter droplets.

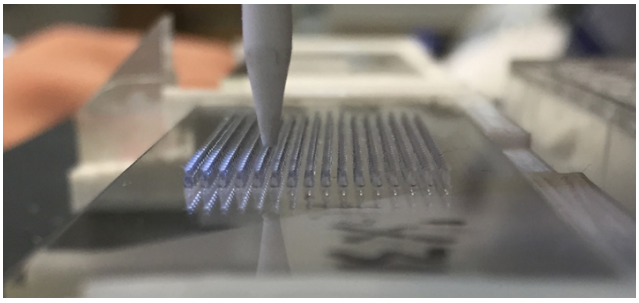

Figure 4. Dispensing rod poised above an nDAP array

4. The High Resolution Imaging Chamber holds an nDAP array enabling imaging of cells isolated within hydrogel droplets. As shown in Figure 5, the chamber is designed to be compatible with confocal microscopes equipped with 63x oil immersion lens.

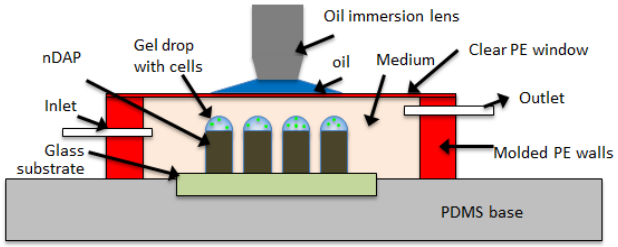

Figure 5. High resolution imaging chamber for nDAPs

2.1 Dispensing Droplets onto an nDAP

Droplets can be dispensed onto the nDAP substrate either in a serial fashion (one at a time) using the DropRbot or all the droplets can be dispensed simultaneously by bringing the nDAP into contact with a reservoir such that all the surface features contact the solution simultaneously.

For serial dispensing, the dispensing tip is brought into contact with either the source drop (Figure 3), a microplate well, or any other reservoir. The rod is retracted, transferring a drop onto the specially engineered rod tip. The dispensing rod is then aligned to an nDAP post and brought into contact with the surface as shown in Figure 6. Because of the design of the surface structures, the alignment between dispensing tip and nDAP post is not critical; misalignment tolerance of ± 100 µm in the x, y and z axes, results in no change to the dispensed volume. As the rod is retracted, the solution remains pinned at the perimeter of the nDAP post. This causes the drop to neck-down, eventually forming an hour-glass shape. Eventually, the elongated drop breaks, transferring a well-controlled volume of solution onto the nDAP surface.

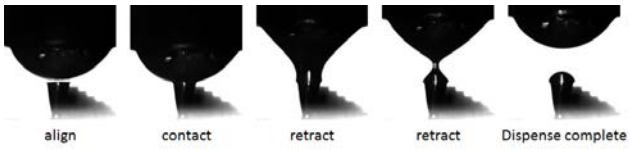

Figure 6. Dispensing process recorded using a high speed camera at 5000 frames per second

The diameter of the nDAP post feature controls the volume of fluid dispensed. A series of nDAP plates were fabricated with the radius of the posts ranging in size from 84 to 356 µm. Water was dispensed on these posts using the DropRbot and droplet volumes were measured. A linear relationship between the post radius cubed and dispensed volume was observed ($R^2 = 0.998$).

3 AIM : ANCHORED IMMERSION MICROPLATE LIDS

AIM lids are comprised of arrays of engineered surface features formed into polystyrene lids that mate with standard 96, 384 and 1536 well microplates; a 96 well AIM lid is shown in Figures 2 and 7.

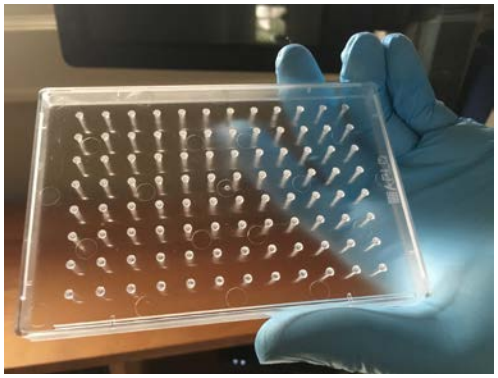

Figure 7. An AIM lid compatible with a standard 96 well microplate. Each post structure can anchor a hydrogel droplet with a volume ranging from 1.5 to 15 µL

Two methods can be used to dispense droplets on the AIM lid posts. Standard pipetting techniques can be used to dispense individual droplets. By controlling the pipette volume, droplets of any size ranging from 1.5 to 15 µL can be dispensed on the surface. For faster dispensing, the AIM lid can be immersed into a reservoir such that all or a portion of the posts are populated with droplets at the same time. A schematic of the parallel dispensing sequence is shown in Figure 8.

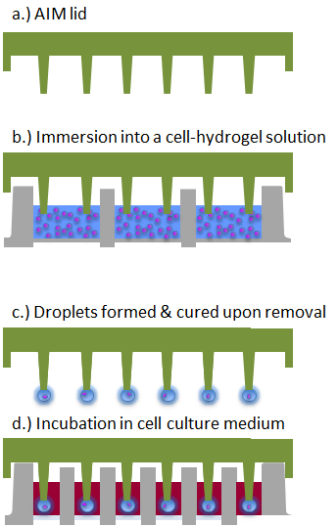

Figure 8. Parallel process for formation of hydrogel droplets containing cells on an AIM lid and culturing in standard microplate.

The lids are fully compatible with high throughput laboratory robots and so the dispensing and handling process steps are easily automated. For research laboratories, ARLD has developed a low-cost benchtop robot that automates this parallel-scale dispensing process.

4 RESULTS

4.1 nDAP plates

The small volume and high density of hydrogel droplets on nDAP plates greatly facilitates the study of interactions between probe molecules and cells using high magnification optical/fluorescence microscopy. For example, HEK 293 cells were dispensed in alginate hydrogel droplets on an nDAP plate comprised of 250 µm diameter posts. The cell density was kept low (5×10^5 cells/mL) so that an average of 2 cells were isolated in each 8 nL droplet as shown in Figure 9a. A live cell dye (FDA, 3 µM) was introduced into the medium surrounding the droplets within the High Resolution Imaging Chamber and images were recorded as a function of time in the confocal microscope (Leica TCS SP8) as shown in Figure 9b-d. The dye rapidly diffuses through the alginate gel and cell membrane. Fluorescence can be seen after only 20

seconds, and the intensity become progressively brighter over the course of 600 seconds.

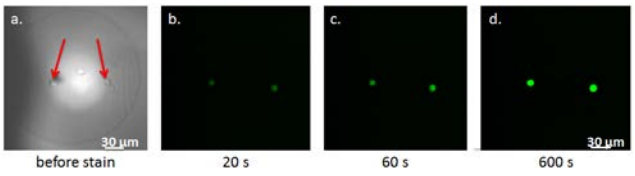

Figure 9. High resoltuion confocal microscope images of HEK cells a.) phase contrast image before staining and fluorescence images after introduction of FDA live cell stain b.) 20 s, c.) 60 s, d.) 600 s.

The alginate hydrogel exhibits similar permeability to peptides. In this experiment, 1MEA liver cancer cells were isolated in 800 nL alginate droplets on 1000 µm diameter nDAP posts. An $(Arg)_9$ cell penetrating peptide, along with a nucleic acid stain (DAPI 50 µM) were introduced into the medium surrounding the cells and imaged using a 63x oil immersion lens. The nucleus and interior of the cell are clearly visible in these high resolution images (Figure 10).

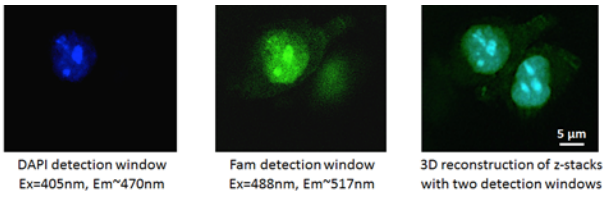

Figure 10. High resolution confocal fluorescence microscope images showing the DAPI, Fam and overlapping detection windows for two 1MEA cells.

4.2 AIM lids

Imaging of cell growth in hydrogel droplets on AIM lids is straightforward as the tools for conventional microplates can be used. For example, cells can be imaged while the droplets are immersed in medium by using clear-bottom microplates and imaging using a live cell microscope set-up. Figure 11 shows images of Jurkat T cells taken at the start and after three days of cell culture at 37 ^{o}C in an alginate hydrogel droplet. By recording images every hour, cell division can be observed with individual cells easily followed.

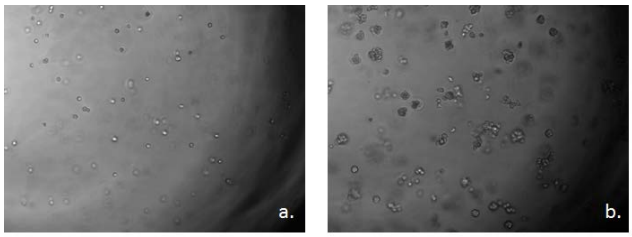

Figure 11. Jurkat T cells cultured in alginate droplets on AIM lid viewed through the bottom of a 96 well microplate using a Zeiss Cell Observer Live-Cell Microscope at (a.) time=0 and (b.) after 3 days

Other standard analysis techniques are also compatible with AIM lids. Fluorescently labeled cells can be quantified using a plate scanner while immersed in the microwell, or the lid can be removed and placed directly on the scanner plate, thereby removing background noise from the medium/solution. Single cell analysis can be performed by immersing the lid supported droplets in microplate wells containing the appropriate solution for dissolving the hydrogel (e.g. EDTA). The released cells can then analyzed using flow cytometry.

AIM lids greatly facilitate long term cell culture because medium can be easily and quickly achieved simply by moving the lid to a microplate filled with fresh medium. There is no risk of losing or damaging the hydrogel droplets anchored onto the substrate. For example, 1MEA liver cancer cells have been cultured in 1% alginate droplets for over 37 days in 96 well plates; 100% of the droplets remain adhered on the AIM lid.

5 CONCLUSION

ARLD has developed two platforms that greatly facilitate 3D cell culture: nDAP and AIM lids. Both platforms use patent-pending surface engineering technologies to achieve highly repeatable (CV < 2%) hydrogel droplets ranging in volume from 0.3nL to 15 µL. The droplets adhere strongly to these surfaces permitting long-term cell culture (> 30 days) without adhesion loss. nDAPs enable the formation of high density droplet arrays (> 500 droplets /cm^2) with volumes as small as 0.3 nL. An imaging chamber enables high resolution (e.g. 63x oil immersion lens) confocal imaging of cells and real-time observation of molecule diffusion. The AIM lid system is seamlessly compatible with all aspects of high-throughput screening assays and equipment. These functional lids securely anchor precise microliter volumes of hydrogels that can be repeatedly immersed into media in standard microplates (e.g. 96 and 384 well) for 3D cell culture and/or compound screening, speeding and simplifying media changes. Cells have been cultured for over 30 days while securely adhered to the AIM lid surface features. Future work will focus on core-shell hydrogel droplets and growth of micro-tissues.

6 ACKNOWLEDGEMENTS

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