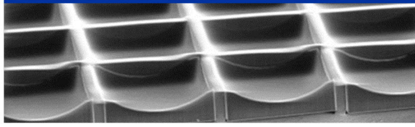

Image, Identify, and Isolate Single Organoids using the CellRaft AIR® System

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Summary

The advancement of three-dimensional (3D) culture systems has transformed cell-based assays for biological research and drug discovery due to their ability to re-capitulate the structure and cellular complexity of in vivo tissues. Organoids, a subtype of 3D cell culture that are grown from stem cells, are unique due to their ability to self-organize and closely replicate in vivo pathophysiology. Traditional culture methods of organoids entail many organoids embedded in a dome or layer of extracellular matrix (ECM). Although efficient in supporting organoid growth, these methods present challenges in being low throughout and difficult to assess due to multifocal imaging requirements, heterogeneity in size and growth rates, and variability within and between experiments. Several laboratory instruments are employed to assess various endpoints using traditional organoid cultures, such as high-content imaging platforms and microplate readers. However, these technologies are generally limited to population readouts rather than assessment of individual organoid responses. These limitations preclude assessment of the heterogeneity within the population or retrieval of single, intact 3D structures for downstream applications.

The CellRaft® Technology developed by Cell Microsystems is uniquely suited to address these challenges in organoid workflows. To enable organoid applications, we developed the 3D CytoSort® Array, software features for imaging and evaluating 3D structures, and user-friendly workflows for culturing hundreds of segregated organoids on a single array. The 3D CytoSort Array and CellRaft AIR® System provide a one-instrument solution for reliable time-course imaging, phenotypic assessment, and automated isolation of single organoids for downstream applications.

The key features of the CellRaft organoid workflow

- *Segregated culture of individual organoids*
- *Temporal imaging of each CellRaft*
- *Phenotypic analysis and isolation of viable, intact organoids*
- *Enables rapid and reliable clonal organoid workflows that are not possible using traditional organoid culture methods*

The 3D CytoSort Array and CellRaft AIR System

The 3D CytoSort Array is a proprietary cell culture consumable that contains over two thousand individual microwells. Each microwell contains a releasable micron scale polystyrene growth surface called a CellRaft (Figure 1A). Together, these microwells enable the culture of hundreds of organoids segregated individually across the CytoSort Array, overcoming one of the greatest challenges in traditional organoid culture methods. Each 3D CytoSort Array has 500 x 500-micron (μm) CellRafts that support organoid growth up to 1mm in diameter. With our user-friendly protocol, organoid-forming cells are seeded in ECM directly onto the 3D CytoSort array at an appropriate density to segment them as single cells, or single clusters of cells, within the microwell chambers.

After sufficient time for matrix polymerization, the 3D CytoSort Array is scanned and imaged in as little as 10 minutes using the CellRaft AIR System, first to identify CellRafts containing single cells, or single clusters of cells, then at desired intervals throughout the experiment to monitor organoid development. The AIR System contains an internal microscope capable of brightfield and three-channel fluorescent imaging (DAPI, GFP, and TexasRed; Figure 2B). The System's software conveniently stores each scan in a central database for each array, allowing for phenotypic image-based analysis of organoids of interest and easy tracking of organoid growth and phenotypic changes over time.

Our proprietary software powers the CellRaft AIR System's functionality for two-dimensional (2D) array scans and Z-stack imaging of selected CellRafts containing organoids of interest. We also developed CellRaft Cytometry™ in our software to automatically identify organoids grown on the 3D CytoSort Array and provide phenotypic assessment of organoids for parameters such as organoid diameter, circularity, fluorescence intensity, and dimensionality. Using CellRaft Cytometry, users can define parameters for each of these metrics and create populations of interest, which can be assigned to specific wells of a 96-well collection plate or saved as gates and automatically isolated for downstream applications.

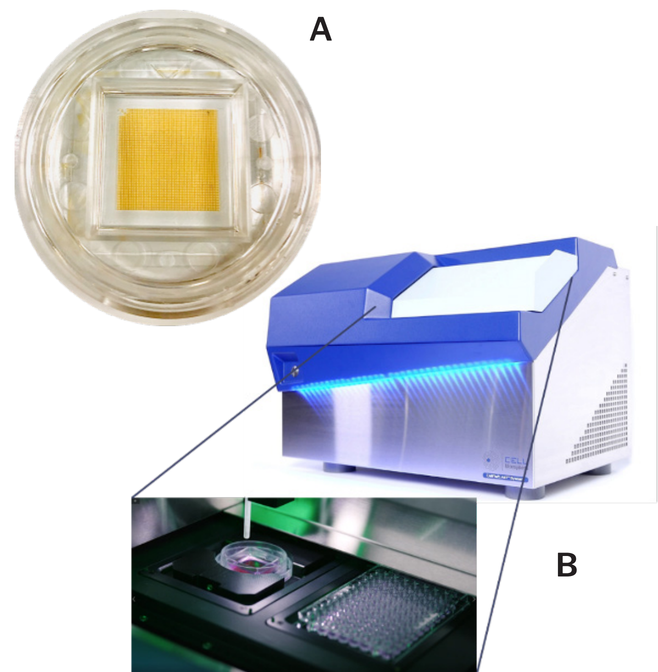

Figure 1: The CytoSort Array (A) and CellRaft AIR System (B) provide fully integrated brightfield and 3-channel fluorescent imaging, automated release and transfer, and optional stage-top incubation.

In addition to imaging, the CellRaft AIR System performs the automated release of CellRafts containing organoids of interest from the CytoSort Array and transfers the CellRaft and organoid to a 96-well collection plate. A motorized needle penetrates the resealable elastomeric floor of the CytoSort Array to displace the individual CellRaft from its microwell. The CellRaft material is loaded with magnetic nanoparticles, allowing it to be retrieved with a magnetic wand. The organoid remains attached to the CellRaft through the transfer, without physical manipulation of

the 3D structure, which permits the transfer of intact, viable organoids for unlimited applications. The Cell Microsystems organoid workflow overcomes many of the challenges of traditional organoid culture methods and provides a one-instrument solution for imaging, analysis, and retrieval of individual organoids, unlocking a new generation of applications for research and drug discovery.

Seeding Cells in Extracellular Matrix on the 3D CytoSort Array

The requirement of ECM for organoid cultures is one of the biggest challenges in the throughput and scalability of organoid assays. Most commercially available ECM products used for organoid cultures require precise, temperature controlled liquid handling due to rapid polymerization at temperatures as low as 10 degrees Celsius. Because of the temperature sensitivity of ECM products and unique design of the CytoSort Array we developed custom, user-friendly cell seeding protocols to ensure cell-matrix suspensions properly wick into the microwells and attach to the CellRafts, steps integral to successful organoid culture.

After preparation of the CytoSort Array (see CytoSort Array User Manual), the array is placed on ice, or at 4°C, for at least 1 hour to cool the array. Once the cell suspension is prepared and counted, the appropriate cell seeding density (recommended starting density is 2,000 fragments, or 5,000 single cells) is pelleted by centrifugation. Cell pellets are resuspended in ice cold growth media with ECM (see CytoSort Array User Manual for ECM recommendations), dispensed on the cold CytoSort Array, and placed on ice for 20 minutes to allow the ECM-cell suspension to settle into the microwells. After the cold incubation, the CytoSort Array is placed in a 37°C incubator to polymerize the ECM for 4 hours prior to the initial scan. This custom seeding protocol is highly efficient and reproducible across multiple ECM products and concentrations, and organoid cell types, and ensures that organoids form within the microwells, loosely attached to the CellRaft surface, which is required for successful imaging and isolation.

Single-Organoid Applications Enabled by the CellRaft Technology

Clonal Organoid Development

The CellRaft Technology excels in clonal cell line development by offering a time, resource, and labor efficient alternative to traditional culture methods, which now extends to clonal organoid culture. Using the 3D CytoSort Array and CellRaft AIR System, hundreds of single organoid-forming cells can be grown spatially segregated on individual CellRafts. Organoids can be imaged over time for clonal verification and organoid development, then isolated intact for downstream propagation. Importantly, organoids can be evaluated for phenotypic and morphologic properties, including fluorescent markers. These properties can be organized and parsed using CellRaft Cytometry, which allows for upstream characterization of the population and isolation of specific single organoids of interest using the AIR System.

To demonstrate clonal organoid workflows, we enzymatically dissociated mouse hepatic organoids, using a DNase I with TrypLE solution, seeded 3D CytoSort Arrays with 5,000 cells in dilute ECM, and performed the initial scan to identify CellRafts containing single cells 4 hours after cell seeding. The array was scanned every 24 hours for 8 days to verify clonality and monitor organoid formation (Figure 2). Using CellRaft Cytometry, sub-population criteria were built to identify CellRafts containing single cells at the initial Day 0 scan and single organoids greater than 50μm in diameter on Day 8. Using the population overlay feature, the intersect of the two populations is easily

viewed and evaluated for desired phenotypic characteristics ahead of isolation using the AIR System. After isolation, organoids continue to grow in dilute ECM in the 96-well collection plates (Figure 3) and can be dissociated into single cells or fragments for propagation of clonal organoids.

The key features of the CellRaft organoid workflow:

- Segregated culture of individual organoids,
- Temporal imaging of each CellRaft,
- Phenotypic analysis, and isolation of viable, intact organoids

This workflow enables rapid and reliable clonal organoid workflows that are not possible using traditional organoid culture methods.

Drug Efficacy and Toxicity Screening

Using organoids for drug efficacy and toxicity screening has the potential to significantly improve the efficiency and outcomes of drug development pipelines due to their physiological relevance, compared to 2D cell-based assays. However, challenges in throughput and assessment of traditional culture methods necessitate the use of advanced instrumentation to design organoid assays that maintain consistency within and between experiments. We adapted the CellRaft Technology to provide a solution for moderate-throughput single organoid screening assays.

One limitation to using conventional bulk culture of organoids for toxicity screening is the variability of organoid number and size between wells in an experiment, and across multiple experiments. We used the CellRaft AIR System and 3D CytoSort Array to culture, image, and isolate single organoids to determine whether the AIR System could be used to create a more uniform organoid assay for toxicity testing. To demonstrate a drug toxicity screening workflow, we treated mouse hepatic organoids with the well-characterized hepatotoxicant acetaminophen (APAP). First, mouse hepatic organoids were dissociated into fragments and seeded on the 3D CytoSort Array (2,500 fragments) in 0.24mg/mL Matrigel (1.2mg total). The array was scanned 4 hours after cell seeding and every 24 hours for 5 days to monitor organoid development. On the fifth day, CellRaft Cytometry was used to identify CellRafts containing two populations of organoids based on organoid diameter, distinguishing variable sized organoids greater than 50 µm from organoids limited to 300-500 µm in diameter. In the 96-well collection plates, single organoids from each collection plate were treated in parallel with a 6-point, 5-fold dose curve of APAP (0.0008-2.5mM) with 5

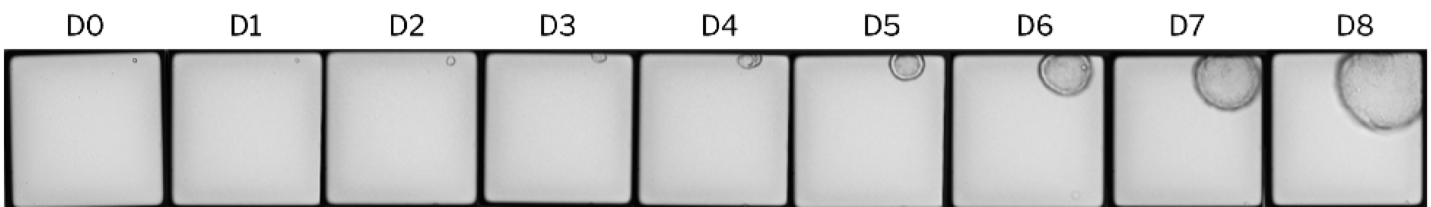

Figure 2: The 3D CytoSort Array and CellRaft AIR System enable temporal imaging and clonal verification of single cell-derived mouse hepatic organoids.

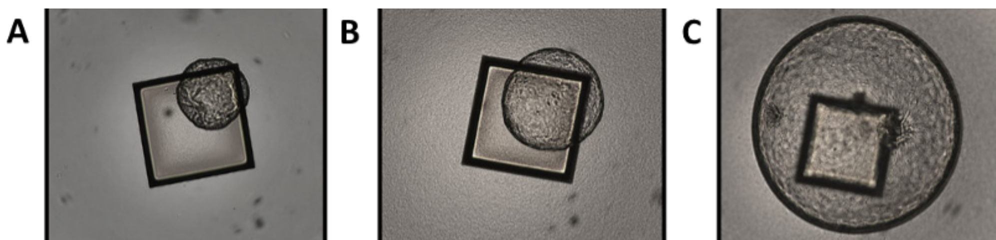

Figure 3: Mouse hepatic organoids isolated from the 3D CytoSort Array continue to grow in 96-well collection plates. Mouse hepatic organoid immediately post-isolation (A), 1 day after isolation (B), and 5 days after isolation (C) in dilute ECM.

replicate wells. For toxicity assessment, kinetic viability was measured every 24 hours for 72 hours using CellTox Green and total ATP was measured after 72 hours of treatment using 3D CellTiter Glo.

Toxicity readouts, such as CellTox Green and CellTiter Glo, are highly sensitive assays that are reliant on cell number. Therefore, to generate significant data, assays must have a high degree of consistency, both within and between experiments for accurate assessment, which is an issue for pooled readouts of many organoids embedded in ECM due to variability in size and number. As shown in Figure 4A, the population of organoids that was not selected for size shows a high degree of variability. This is directly correlated to large error bars in the highest dose of APAP after 72 hours of treatment, and across all doses for the 72-hour ATP readout (Figure 5A). By using CellRaft Cytometry to identify organoids from a narrower range of size, we are able to isolate and evaluate a more consistent population of organoids (Figure 4B). Because of this, the replicates for each dose demonstrate less variability in both viability and ATP readouts (Figure 5B). The dose curve from organoids 300-500μm in size was sufficient to calculate an ED 50% (0.6mM), which was not possible in the dose curve using inconstant sized organoids. Altogether, these data demonstrate the ability to use the CellRaft Technology and CellRaft Cytometry for developing more consistent, reliable organoid

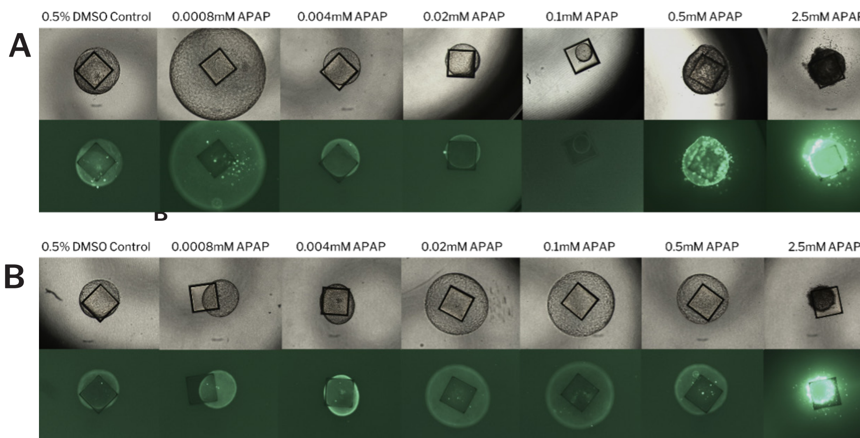

Figure 4: Representative images of organoid size variability of mouse hepatic organoids in each Acetaminophen (APAP) dose curve from variable sized organoids >50μm (A) and organoids limited to 300-500μm (B) after 72 hours of treatment. Each CellRaft is 500 x 500μm in diameter.

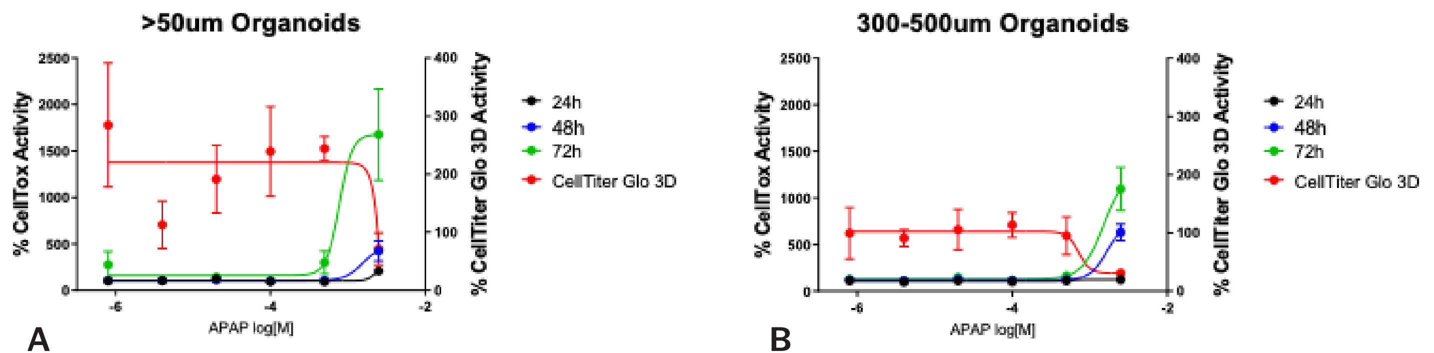

Figure 5: Dose-response curves for mouse hepatic organoids treated with a 6-point dose curve of Acetaminophen (APAP; 0.0008-2.5mM) with 5 replicate wells of each treatment for 72 hours. The left y-axis shows 24 (black), 48 (blue), and 72 (green) hour CellTox activity relative to the average DMSO control wells. The right axis shows the percent CellTiter Glo 3D activity relative to the DMSO controls wells after 72 hours of treatment.

screening assays for drug efficacy and toxicity within and between replicate experiments.

iPSC-derived Organoids

Organoids derived from human induced pluripotent stem cells (iPSCs) have become valuable methods in studying development and disease, because they offer the ability to understand self-organization, tissue-specific differentiation, and are amenable to genome editing using CRISPR technology. However, iPSC-derived organoid workflows are not trivial and often require many re-plating steps to achieve tissue-specific differentiation. Closely monitoring phenotypic changes of individual structures throughout the process of differentiation is not possible using bulk organoid techniques to generate iPSC-derived organoids, due to such re-plating step. We adapted the CellRaft Technology to not only simplify iPSC-derived organoid workflows but also demonstrated the ability to closely monitor differentiation of hundreds of individual organoids on a single array.

We used iPSCs with a RFP-positive for β-actin reporter (Sigma Aldrich; cat #IPSC1028) and commercially available kits to differentiate iPSCs into choroid plexus and kidney organoids (StemCell Technologies, cat #s 100-0824 and 05160, respectively). Two 3D CytoSort Arrays were seeded with 5,000 small fragments of iPSCs using the protocol described above. For choroid plexus organoid differentiation, media for embryoid body formation was used for cell seeding whereas iPSCs for kidney organoid differentiation were seeded in iPSC expansion media, per the manufacturer's guidelines. Each array was scanned 4 hours after cell seeding, and at least every 24 hours thereafter to monitor phenotypic changes throughout the differentiation process.

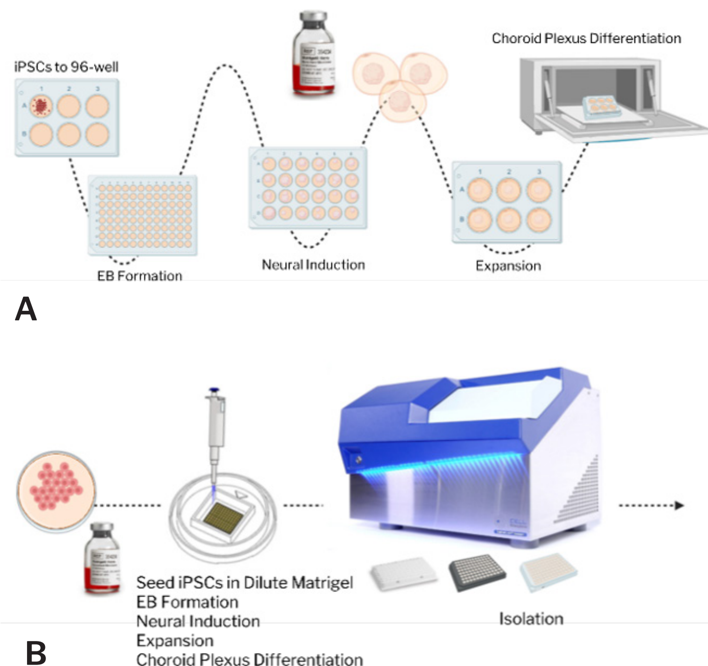

Figure 6: A. The standard protocol for iPSC-derived choroid plexus organoids (A) involved multiple replating steps and media changes throughout differentiation. B. Using the 3D CytoSort Array, we simplified the workflow by seeding cells directly onto the array and performing media changes at specified time intervals. Using the CellRaft AIR System provides time course imaging of differentiation and organoid isolation at desired timepoint throughout differentiation.

The standard protocol for choroid plexus organoid differentiation involved multiple replating steps throughout the differentiation process (Figure 6). We eliminated these replating steps by directly seeding the iPSCs in dilute ECM and simply performing media changes at the recommended time intervals for each step of the differentiation process. This simplified workflow allows for reliable imaging of hundreds of organoids throughout the process of differentiation (Figure 7). Choroid plexus organoids were maintained on the 3D CytoSort Array for 30 days with media changes every 48-72 hours. In addition, CellRafts containing organoids of interest can be isolated from the array at desired stages of differentiation

for characterization using techniques such as transcriptomics.

Similarly, iPSC-derived kidney organoids were generated by seeding iPSCs on the array, allowing for iPSC colony expansion and media changes were performed to achieve kidney organoid differentiation (Figure 8). In addition to 2D scans, at desired time points, organoids were selected for z-stack imaging providing morphologic data through the full height of the 3D structures. Using 2D and z-stack imaging features, organoids can be analyzed for characteristics such as fluorescence intensity. For further evaluation, single organoids can be isolated from the array at desired time intervals throughout the differentiation process

Conclusions

The Cell Microsystems organoid workflow demonstrates how the 3D CytoSort Array on the CellRaft AIR System with CellRaft Cytometry provides a one-instrument solution, overcoming challenges in traditional organoid culture workflows. The 3D CytoSort Array and user-friendly workflows enable the culture of hundreds of organoids segregated individually across the CytoSort Array, each of which can be imaged over time using the CellRaft AIR System. In addition, single organoids grown on the CytoSort Array can be analyzed for user-defined phenotypic characteristics with CellRaft Cytometry. This combined with automated isolation and recovery of single intact organoids unlocks the ability to use organoids in a universe of applications, including clonal cell line development, drug development and toxicology, genomics, and gene therapy.

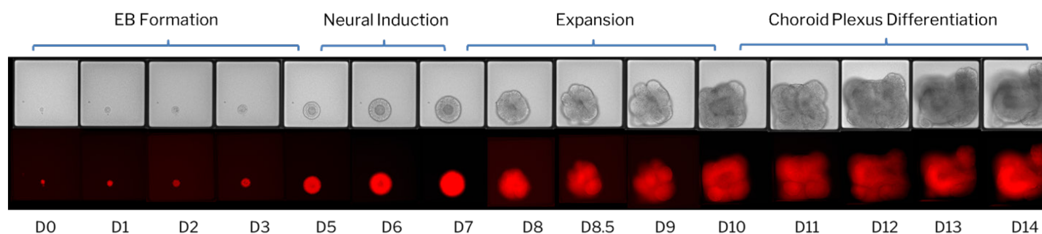

Figure 7: iPSC-derived choroid plexus organoids grown on the 3D CytoSort Array. RFP-positive iPSCs were seeded on the 3D CytoSort Array in dilute ECM and imaged throughout the process of choroid plexus organoid differentiation.

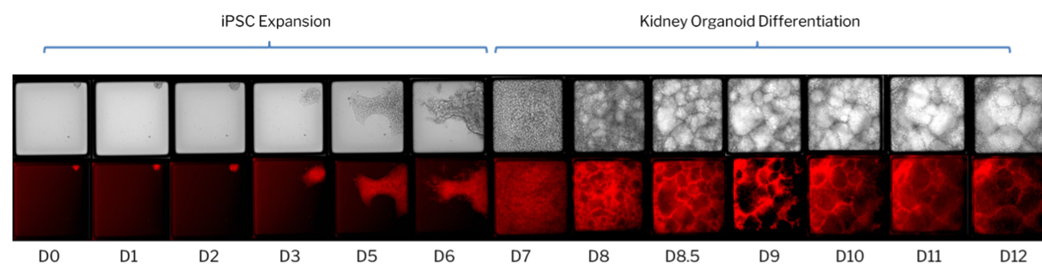

Figure 8: iPSC-derived kidney organoids grown on the 3D CytoSort Array. RFP-positive iPSCs were seeded on the 3D CytoSort Array in dilute ECM and imaged throughout the process of kidney organoid differentiation.

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