



SpAi: A machine-learning supported experimental workflow for high-throughput spheroid production and analysis

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ABSTRACT

Three-dimensional (3D) cell cultures, especially spheroids, provide a physiologically accurate model for cancer research in comparison to conventional two-dimensional (2D) cultures. Nevertheless, the difficulties in producing and analyzing spheroids have impeded their extensive use in high-throughput screening—a critical process for drug discovery. This study presents a simplified process for the effective creation and examination of spheroids using a 3D-printed mold casted polydimethylsiloxane (PDMS) microwells. The utilization of our specially constructed mold facilitated the creation of consistent spheroids, which were subsequently exposed to doxorubicin for the purpose of anticancer medication treatment. We herein improved spheroid analysis by including a convolutional neural network (CNN) model, specifically U-Net, into a graphical user interface (GUI). This integration allows for automated detection and measurement of spheroid size from microscope pictures. The performance of this system surpassed that of conventional image analysis methods in terms of both accuracy and efficiency. The implemented workflow offers a scalable and cost-efficient platform for conducting high-throughput drug screening, which has the potential to enhance the success rates of cancer therapies in clinical trials.

1. Introduction

Individual cells are organized in human body into complex structures inside the tissues to operate various metabolic tasks. This organization is mainly driven by the support of extracellular matrix and the internal cellular components to form complex 3D structures that can interact with their close environment from many dimensions via different mechanisms. However, in order to gain insight on the failures in these cell-to-tissue systems, majority of the studies utilize 2D cell cultures in laboratory settings. Although 2D cell cultures are effective strategies to grow, maintain and test various types of eukaryotic cells that are attached to the growth environment, they lack representing the essential morphologic and biological properties of the tissue structures.

The constraints of 2D culture systems in screening chemical compound libraries and discovering drugs contribute to well-documented

inefficiencies in identifying compounds that effectively transition to clinical oncology (Hutchinson and Kirk, 2011). Despite substantial investments of time and resources in developing new cancer treatments, therapies showing promising results in preclinical tests still face a failure rate as high as 90% in clinical trials (Sun et al., 2022). The low productivity greatly escalates the expenses of cancer medication development to approximately \$2 billion for each individual drug (Pillaiyar et al., 2020), (Lindsay, 2003), (DiMasi et al., 2003). Increased adoption of in vitro models that closely mimic real human tumors would aid in the identification of safe and effective compounds, hence lowering expenses and time spent on compounds that prove unsuccessful in later stages of drug development.

To overcome these limitations and achieve a physiologically relevant cell culture for disease modelling studies, 3D cell cultures have been developed with various fabrication methods for producing culture

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vessels (Jensen and Teng, 2020). Essential features of a 3D cell culture system could be defined as a cell growth environment that prevents cellular attachment to surfaces, while facilitating cell-cell attachment and the formation of cellular aggregates, known as spheroids, which mimic the 3D structure and behavior of tissues *in vivo*. This basic property of forming cellular clumps originates from natural properties of the cells, which is also the main mechanism that drives tissue and organ formation during the developmental stage. Various strategies have been developed to produce spheroids, which include the earliest method known as hanging-drop technique, and recent techniques based on engineering and fabrication of non-adherent surfaces (Lee et al., 2019). Among these techniques, 3D printing technology offers significant advantages in terms of time efficiency, cost-effectiveness, and high customizability for producing microwells, which are small, well-like structures designed to support the formation and growth of spheroids. For instance, while photolithography and deep reactive ion etching (DRIE) are widely used microfabrication techniques (Pimpin and Srituravanich, 2012), they are expensive, labor-intensive, and require specialized facilities such as a cleanroom. On the other hand, 3D printing technology provides high control fabrication in micrometer levels, which are the essential size for microwell production. Moreover, microwells with various design parameters (geometry, material composition, surface properties, etc.) can be easily customized using 3D printing technology. This will facilitate the production of multiple spheroids with a homogeneous size and shape distribution, and eventually provide a high level of consistency in experiments (Decarli et al., 2022). On the other hand, in microwell strategy, anti-adherent materials or coatings are applied to the non-toxic, cell-culture-grade materials, which are molded or grafted into microwell shapes with desired parameters.

Recently, there have been notable progressions in the development of more intricate 3D models (Hsiao et al., 2009), (Weigelt et al., 2014). Such models, ranging from a few tens of micrometers to millimeters, more realistically replicate the original tumor environment and offer superior platforms for studying cancer biology and drug discovery. Cancer cell spheroids serve as a simple and efficient *in vitro* model for investigating solid tumors (Friedl and Wolf, 2003). These structures are created as a result of the inherent capability of cancer cells in the epithelial tissue to develop connections between cells and organize themselves into dense clusters on non-adhesive surfaces or within three-dimensional frameworks like scaffolds or matrices (Paschos et al., 2009), (Nunes et al., 2019). They exhibit a 3D structure that closely resembles avascular tumors, which lack blood vessels and rely on continuous diffusion of oxygen and nutrients (Lin and Chang, 2008). They possess strong cell-to-cell and cell-to-ECM adhesions, uneven exposure to soluble substances, and a hypoxic central region with inactive cells, which promotes the development of cancer stem cells capable of regenerating tumors and resisting pharmaceutical interventions (Legate et al., 2009) (van Dijk et al., 2013) (Shabana et al., 2018), (Borah et al., 2015). Furthermore, the acidic extracellular tumor environment affects cancer cell processes and contributes to chemotherapy resistance by reducing the absorption of weakly basic drugs like doxorubicin (Vivanco and Sawyers, 2002) (Shabana et al., 2018). The analysis of tumor spheroid studies from microscope images, particularly those involving a high number of spheroids, can be a tedious task prone to user bias, especially when non-automated systems are used. Image analysis techniques are commonly employed in spheroid analysis to supplement microscopy imaging and obtain quantitative data, such as the diameter or volume of the spheroid. These data can be used to calculate the number of cells or to identify general growth trends in response to various stimuli, such as pharmaceuticals (Costa et al., 2016). Incorporating machine learning into the image processing pipeline offers a more effective and efficient chance at bulk image analysis. Recently, image analysis tools enhanced with machine learning and artificial intelligence algorithms have become mainstream in biological research.

Various studies have employed computer vision and machine learning reliant tools for the detection and analysis of spheroids (Vaidyanathan et al., 2021), (Grexa et al., 2021), (Chen et al., 2021). Among these, U-Net, a convolutional neural network (CNN)-based algorithm gained attention in recent studies since U-Net provides pixel-level sensitivity for detailed segmentation. Besides, U-Net's ability to skip connections for connecting layers directly provides better preservation of spatial details in images that could be lost during down-sampling. More importantly, U-Net is suitable for small datasets which makes U-Net a good candidate for image processing studies that have unique data sets or that are designed to be operated with a limited number of measurements. Different studies incorporated U-Net or different variations of neural network-based image analysis tools to support high-throughput image analysis efforts (Kahveci et al.). Half-UNet variant was developed for a simplified encoder-decoder scheme to achieve similar segmentation accuracy with U-Net by using less parameters and floating-point operations for endocardium and epicardium identification (Lu et al., 2022). In a similar study, a light-weight U-Net architecture, Mu-Net, was developed with small brain organoid image datasets with faster computation time and protected accuracy (Brémond Martin et al., 2023). Not only for analysis of 2D image data, U-Net could also be trained for 3D image segmentation and could be used to analyze brain tumor magnetic resonance imaging (MRI) data (Mehta and Arbel, 2019). Despite this, the current literature lacks a unified strategy that offers a comprehensive approach to producing and analyzing spheroids efficiently. Developing an open-source experimental workflow that integrates these advanced tools could significantly improve the accuracy and efficiency of spheroid analysis (Mitrakas et al., 2023).

In this study, we designed and built a 3D-printed mold to produce polydimethylsiloxane (PDMS) microwells in pyramidal shape to obtain an optimal and multiplexed spheroid production microwell system for high-throughput experiments. Our mold structure could hold 228 spheroids in a single multiwell and can be imaged completely with 4-picture per multiwell application. In addition, we adopted our in-house spheroid formation protocol to the developed microwell system for obtaining an optimized protocol for future studies. After that, we tested our spheroid production system with a case study, in which we tested we tested effect of Doxorubicin, a very well characterized cancer drug, on MCF7 human breast cancer cell line. As a final step, we developed an open source GUI for spheroid analysis, which identifies 99.9% of spheroids on a single image, numbers spheroids accordingly and measures individual spheroid sizes accordingly.

2. Materials and methods

2.1. Fabrication and characterization of spheroid molds

Spheroid molds for pyramid-shape wells were designed using Shapr3D software as illustrated (Fig. 2A). Each mold was designed to harbor 228 wells with 460 μm in depth and 822 μm in width. Mold printing process was operated with a Formlabs 3D printer using a standard clean resin with 2.2s time exposure, 12s time bottom exposure, and 25 μm Z-axis printing resolution parameters. After printing, each mold was cleaned up with isopropanol for 2 min, and cured under 405 nm UV light for 30 min. For the formation of PDMS wells, molds enclosed from the base with kapton tape to provide the reservoir for PDMS casting. PDMS and curing agent was mixed with 10:1 (w/w) ratio, and then degassed under vacuum for 1 h and cured at 80 $^{\circ}\text{C}$ for 2 h. Characterization of PDMS microwells was operated using a scanning electron microscopy (SEM) and 3D laser scanning microscopy (KEYENCE VK X100) to analyze the aimed and obtained features of microwells. In these analyses, PDMS surface was coated with 10 nm of gold: palladium (Au: Pd).

2.2. Cell culture

DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) medium was used for MCF-7 breast cancer cells. Additionally, 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin were added to this medium for MCF-7. Cells stored at -80°C were quickly thawed in a 37°C water bath for the culture process. After thawing, 5 mL of fresh medium was added to the different tubes containing the cells. The prepared tubes were centrifuged at 1500 rpm for 5 min to pellet the cells, and the supernatants were discarded. This step removed the DMSO used for cell freezing from the medium. The cell pellets were resuspended in 5 mL of fresh medium and suspended cells added to a 25 cm^2 cell culture flask. The cultured cells were incubated at 37°C with 5% CO_2 . The mediums were changed every two days. When the cells covered 80% or more of the culture flask surface, passage was performed. For the passage process, the medium was removed, and the cells were washed with PBS. After washing, 1 mL of trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA) was added, and the cells were incubated in a 37°C incubator for 5 min. After incubation, 3 mL of fresh medium was added to the cell culture flask, and the passage process was completed at a 1:5 subculture ratio.

2.3. Formation of spheroids

Formation of spheroids was operated as explained before (Ismail et al., 2024). Briefly, MCF-7 cells were initially cultured in tissue culture plates. Following this, the cells were transferred to microwells for spheroid formation. PDMS microwells were fabricated and sterilized prior to use in cell culture experiments by autoclaving at 121°C for 15 min. Sterilized, pyramid-shaped PDMS-based microwells were then placed into a sterile 12-well plate. To reduce background noise in subsequent microscopy studies during spheroid formation, a transparent medium devoid of phenol red was prepared. For MCF-7 cells, 45 mL of FluoroBright DMEM (Gibco) was mixed with 4.5 mL (10%) heat-inactivated fetal bovine serum (FBS), 450 µL of PEN-STREP (Gibco), and 450 µL of Glutamax L-Glutamine (Gibco).

To prevent cell adhesion to the PDMS microwells and promote spheroid formation, the microwells were rinsed with an anti-adherence rinsing solution. The PDMS molds were first washed with 500 µL of anti-adherence solution. The 12-well plate was centrifuged at 1000 rpm for 5 min. After centrifugation, the solution was removed and the PDMS microwells were rinsed with 500 µL of the sterile medium. Cell passage was performed, and the cells were counted to ensure the correct seeding density. Based on the cell count, 456,000 cells (2000 cells/microwell) were added to all microwells combined inside a single spheroid mold. The volume in each microwell was adjusted to 500 µL with fresh medium. The 12-well plates were centrifuged at 2500 rpm for 5 min to allow the cells to settle into the microwells. To ensure adequate medium for spheroid formation, an additional 700 µL of medium was slowly added to the PDMS microwells without disturbing the settled cells.

2.4. Anticancer drug treatment

Doxorubicin anticancer drug was administered to MCF-7 cells at 0.025 mM and 2.5 mM doses. 4 days after the spheroids were formed, doxorubicin was added to the wells and the cells were monitored for 12 days.

2.5. Spheroid imaging

EVOS M5000 microscope was used to image the spheroids. Images were acquired at regular intervals (every 4 days, from day 0 to day 12) to monitor spheroid growth and response to Doxorubicin treatment. To minimize variability, all images were taken under consistent lighting conditions (EVOS M5000 $\times$, 1.25 $\times$ magnification, Light adjustment: 0.100), and each mold was divided into four quadrants to ensure

representation of spheroids from different parts of the well. This approach aimed to capture a diverse range of spheroid shapes and sizes, as spheroids may potentially behave differently depending on their location within the microwells.

2.6. Machine learning and computation

One widely used algorithm in computer vision-based applications is the convolutional neural network (CNN). CNNs excel at tasks like image classification, segmentation, and object detection (Zhao et al., 2024). The U-Net architecture is a CNN primarily designed for biomedical image processing applications (Ronneberger et al., 2015). It was chosen as the architecture to detect the spheroids from the microscope images.

2.7. Detection of individual spheroids from microscope images using U-Net

U-Net was trained to detect the spheroids from the microscope images using semantic image segmentation (Fig. 4D). The images are processed using two labels: *spheroid* and *not a spheroid*. The pixels corresponding to the *spheroid* label become white and the ones corresponding to the not a spheroid label become black. Once a raw microscope image is inputted into the trained U-Net algorithm (Fig. 4A) it will output a segmented version where only the detected spheroids are present (Fig. 4B). Layers of the implemented U-Net architecture used as given in Table S1.

The first stage of training U-Net is the preparation of the dataset. For this task, Labelbox a web-based tool that provides various methods for dataset creation, including image segmentation, was used to manually segment the images. Each segmented image was then paired with its raw counterpart to generate the dataset. The dataset, consisting of 291 images with their segmented counterparts, was split into 70%-10%-20% where 70% of the raw/segmented image pairs were used to train U-Net, 10% was used for validation during training, and 20% were used to test it afterwards. To evaluate the efficiency of training, 5-fold cross validation was performed in which the dataset was randomly separated into 5 different groups of training and validation data with a separate test data corresponding to %20 of the dataset.

U-Net was implemented using the Keras framework (which is now a part of Tensorflow). Programming was done using Python 3.10 on the Spyder integrated development environment.

2.8. Spheroid size calculations from U-Net outputs

The boundaries of the spheroids in the segmented images outputted by U-Net are detected by using OpenCV's *findContours* function which is based on a border following algorithm (Suzuki and Abe, 1985). A circle is then fitted around each spheroid in the filtered segmented image (Fig. 4C) to determine its diameter, measured in pixels. To convert these pixel-based diameters into real-world units, the dimensions of each pixel have to be defined. This is achieved by labeling and training a separate U-Net Model for just detecting the scale lines. The Hough transform (Duda and Hart, 1972) is then applied to the segmented scale line on the original microscope image. Once the pixel dimensions are calculated, in micrometers (µm), they are used to convert the diameters of the detected spheroids into physical measurements.

2.9. Graphical user interface implementation

The finished algorithms were compiled into a graphical user interface (GUI) enabling users to rapidly analyze up to four spheroid microscope images simultaneously. The name SpAi, a combination of *spheroids* and *artificial intelligence* was chosen as the name of the software. Each image section of the GUI made to have four buttons. The *Upload Image* button enables the user to upload a microscope image containing spheroids from any location in their computer. The *Analyze*

button runs the detection algorithm over the uploaded image. For the cases where manual elimination (e.g. due to faulty labelling) is required, an *eliminate* button was also included into the GUI. In the analysis, users can first click on the highlighted contour they want to eliminate. The number of the selected contour is shown below the image. Afterwards, upon clicking the *eliminate* button, the faulty detected contour will be erased. After elimination, the spheroids are automatically renumbered. A step-by-step guide showing the occurrence and elimination of faulty detections can be found in the supplementary (Fig. S2).

For the case of accidental or unwanted spheroid contour removal, a *reset* button is also placed in the GUI. In the final step, the *Data Extraction* button on the bottom left corner of the GUI utilized to generate an Excel worksheet (.xlsx) file showing the size of each of the numbered detected spheroids.

3. Results and discussion

Fig. 1 shows the complete SpAi workflow used in this study. Mold models were obtained after 3D design as illustrated in Fig. 2A, and PDMS casting with this model resulted in clear spheroid formation molds (Fig. 2B). Characterization of cast microwells made by first using SEM imaging (Fig. 2C). The resulting image shows formation of pyramidal-shape microwells with a blunt end after the printing. This result could be interpreted as the capillary action of the tips and cured artifacts of resin at the tip points. In order to measure the obtained 3D structures, 3D laser scanning microscope (Keyence) was used. As a result 818 μm depth and 423 μm width were measured in single microwell feature, which shows 1% error for depth and 8% error for width (Fig. 2D). Since the overall pyramidal shape is not affected, the obtained microwells are used for spheroid formation throughout the work. Details on both PDMS microwell and the 3D printed microwell production mold dimensions and design are given in Fig. S1.

In order to train the U-Net for spheroid size measurement, a custom dataset consisting of 291 raw images paired with their segmented counterparts were used. U-Net was trained over 58 epochs with the best performance observed at the 42nd epoch. The 5-fold cross validation results can be seen in Table 1. The average training loss and testing accuracy were found to be 0.0828 and 0.894 respectively. The training and testing performance of U-Net can be further improved in the future with a dataset containing a higher volume of samples.

In order to observe the effect of processing power required for training U-Net from scratch, two different computers were used. The first utilized a 13th generation Intel® Core™ i9-13980HX CPU, and the latter used a 6th generation Intel® Core™ i7-6700HQ CPU. In both setups, training U-Net from scratch was achieved in under 2 h, pointing out that with the current dataset, a single computer with an i7 or above CPU is sufficient to train U-Net. More expensive high-power training systems (e.g., web servers) were not required in this scenario. Using more computationally expensive hardware for training U-Net can be considered if the dataset is broadened to incorporate images of spheroids consisting of different cell lines, imaging conditions (e.g. fluorescent

images, images taken with different microscopes), or experimental values (different pharmaceuticals introduced).

Using more computationally expensive hardware for training U-Net can be considered if the dataset is broadened to incorporate images of spheroids consisting of different cell lines (e.g. A549 cells), imaging conditions (e.g. fluorescent images, images taken with different microscopes), or experimental values (different pharmaceuticals introduced).

Despite the 89.4% accuracy of U-Net, the algorithm is still in development. Possible faulty detections may arise from optical noise from the medium, shadows, or even potentially from contamination. U-Net can unintentionally detect these as spheroids and segment them. These are then passed onto the GUI as detected spheroids. In order to tackle these challenges, a manual elimination mode was included to the GUI, enabling the users to easily remove potential errors. Examples of the detection of such errors as well as their manual elimination are shown in the step-by-step user guide in Fig. S3.

To illustrate the effectiveness and efficiency of SpAi, three sample images (Fig. S2A) were processed using four alternative algorithms that require less computational processing compared to U-Net training (Fig. S2). In the first one, using MATLAB, the images were blurred (Fig. S2B) then processed using Canny edge detection (Fig. S2C). As demonstrated in Fig. S2C, the detected edges in the spheroids do not form full contours and there are lots of false detections arising from noise. Using the circular Hough transform as an alternative method fails at detecting most spheroids in the first image (Fig. S2D). Moving on to ImageJ's automated tools, automatic segmentation with Li (Fig. S2E), mean (Fig. S2F), and Otsu (Fig. S2G) thresholding were applied to the image. In all three cases, while successful at detecting most spheroids, the ones at the edges of the mold were detected as single large entities with the edges of the mold or incorrectly shaped due to shading. It should also be noted that while these methods can partially detect spheroids, they also require additional manual post-processing steps to isolate, count, and measure each detected spheroid identification tasks that SpAi performs automatically (Fig. S2H). Moreover, these post-processing steps multiply as the number of spheroid images increases, whether due to more samples or repeated imaging over time.

To further address the effectiveness and efficiency of SpAi, a sample of the data was also processed on ImageJ. Two different versions of image segmentation were applied to the test image (Fig. 3A): Manual fitting and automated segmentation. In manual fitting, a circle is fit over a spheroid within the image (Fig. 3B, closeup can be seen in Fig. 3C). While this is an effective way to detect and measure the diameter of a spheroid, using this method individually for every spheroid in all the images will be time consuming, especially for datasets which include hundreds of spheroids. For the second ImageJ method, a two-step process was attempted for automated segmentation. First, in order to reduce noise and unwanted detections (e.g. the borders of the wells) binary thresholding was applied to the raw image (Fig. 3D). This was followed by ImageJ's automated segmentation function which performed poorly at detecting the spheroids (Fig. 3E). As can be seen from Fig. 3E, the perimeter of the mold has been detected alongside the left walls of the

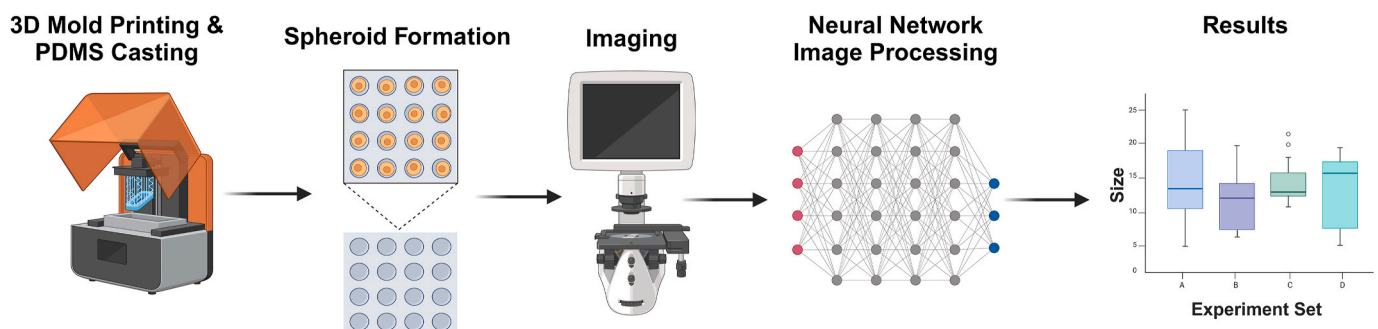


Fig. 1. SpAi workflow. Created by Biorender.

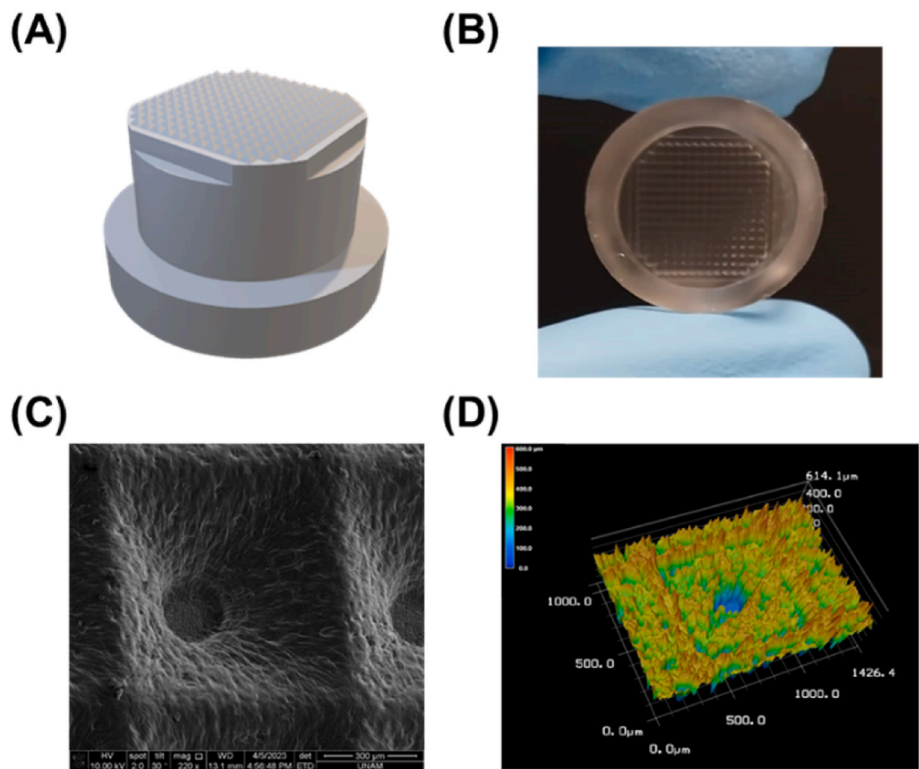


Fig. 2. (A) 3D Spheroid microwell negative mold design, (B) PDMS microwell, (C) SEM image of single microwell, (D) Keyence image and depth/width measurement of single microwell.

Table 1
5-fold cross validation results for SpAi’s U-net.

Fold #	Training Loss	Accuracy
1	0.085	0.89
2	0.073	0.90
3	0.074	0.90
4	0.088	0.89
5	0.094	0.89
Average	0.0828	0.894

spheroids in the bottom left column. Most spheroids in the image have not been detected and measuring the perimeter from the ones that are partially detected will be arduous if not impossible. SpAi’s segmented output of the same image (Fig. 4A and B) show all the spheroids detected and makes measuring their diameters possible (Fig. 4C). SpAi’s GUI also allows direct and rapid extraction of the spheroids size information which cannot be done using ImageJ (Fig. 5A-C).

In order to gauge the performance of SpAi, ImageJ was used to manually measure the radii of a total of 528 spheroids across ten microscope images. These values were then compared to their counterparts generated using SpAi. The results are presented in Table 2. Across the ten images, SpAi’s output shows close values to the means of the spheroid radii when compared to the values obtained using ImageJ. The biggest difference was in the first image, where the difference was less than 10%. The standard deviation for the radii values is also close between SpAi and ImageJ, indicating consistency in measurements. The average mean absolute error (MAE), which shows the absolute difference between the radii values of SpAi and ImageJ, was calculated to be 15.12 μm , which is below the average standard deviation values obtained using ImageJ (31.40 μm). This indicates that SpAi’s errors in estimation are within acceptable limits. The average root mean square error (RMSE) values, which penalize larger errors more than MAE, were found to be 20.14 μm . Finally, the average of the mean absolute percentage error (MAPE), which was implemented to establish the errors as

percentages, yielded 6.40% for the ten images. Individually, the highest errors were observed in image 1 with a MAPE of 14.63%. These relatively low percentage errors suggest that SpAi provides an overall reliable approximation of spheroid radii, with the highest error still remaining under 15%. These error metrics show that SpAi performs well in comparison to manual measurements performed using ImageJ, with error rates generally within acceptable ranges. This suggests that SpAi is a promising tool for automated spheroid radius measurement. In high-throughput drug screening studies based on machine learning tools, 90–100% accuracy is generally accepted for primary screening studies, although human expert accuracy level is expected to be between 95 and 98%. From average MAPE across all images, SpAi’s accuracy could be derived as 93.6% which will be suitable for general screening studies as a machine learning tool. Since SpAi has a manual image curation ability, this will be increasing the accuracy further in real world applications. Variation in error rates in images are measured to be ranging between 2.89% and 14.63%, which could be considered high among image sets, but could be reduced with increasing training data set. However, there is still some room for improvement, which could be achieved by training SpAi with a larger dataset.

In order to test the SpAi GUI, an experimental setup designed with spheroid formation started with the seeding of MCF-7 cells (human breast cancer cell line) to the produced microwells, and separating 3 replicates for each experimental setup. Here, a control group was retained as 3 separate molds with 213 wells for each and 3 separate molds for 0.025 mM and 2.5 mM Doxorubicin induction for MCF-7 cells. Doxorubicin dose was selected by considering dose-effect of the drug studied previously in the literature (Lovitt et al., 2018). For imaging spheroids, each mold separated into 4-pieces (upper-left, upper-right, lower-right and lower-left) for visualization at 1.25X magnification. Spheroid response to the Doxorubicin was recorded as light microscopy images from day 0 to day 12 with a 4-day period. SpAi was then used to track the size of these spheroids by generating.csv files of the radii of these spheroids for each imaged day. As a result of the experiment, 6–8%

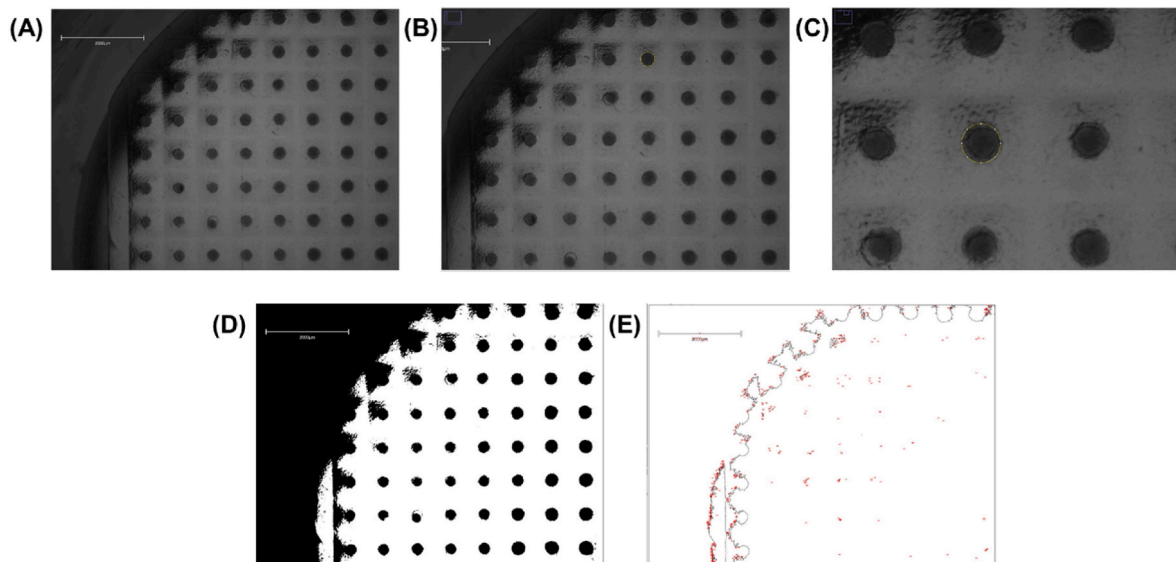


Fig. 3. (A) The raw microscope image of the upper left quadrant of a well containing spheroids, (B) Manual segmentation applied to a single spheroid in the center of the second row using ImageJ, (C) Closeup of the manually segmented spheroid from 1B, (D) ImageJ's thresholding function applied to the raw image in order to decrease noise and simplify automated segmentation, (E) The output of ImageJ's automated segmentation applied to the raw image.

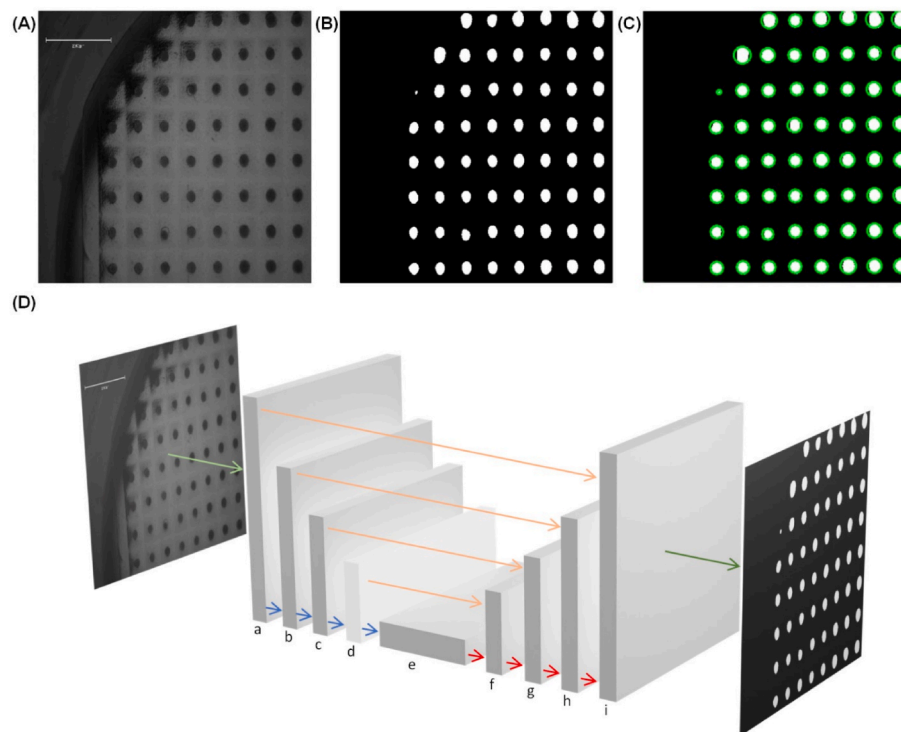


Fig. 4. (A) The raw microscope image of the upper left quadrant of a well containing spheroids. (B) The U-Net outputted segmented image form of the microscope image consisting of two regions: white for spheroids and black for everything that is not a spheroid. (C) Circles fitted onto the segmented spheroids of the mask outputted by U-Net. (D) A visualization of the implemented U-Net architecture. The implemented U-Net takes raw microscope images as inputs and outputs their segmented forms. The blue arrows represents the contracting path where the image is passed through multiple convolutional and max-pooling layers (a–d) reducing the spatial dimensions of the image while increasing the number of features. The red arrows represents the expanding path where spatial dimensions removed in the contracting path are restored. The layers in this path (f–i) contain convolutional and up-convolving layers. The orange arrows represent skip connections between layers of the same size which improves spatial information recovery. Finally, layer e is the bottleneck layer which connects the contracting and expanding paths. Detailed information about the implemented U-Net architecture can be found in the supplementary. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

shrinkage in median spheroid size between day 8 and day 12 measured successfully for 0.025 mM Doxorubicin-induced experimental set, which was not a case for control group in MCF-7 cells (Fig. 6A). In contrast, 2.5

mM Doxorubicin induced group show no decrease in growth trend (Fig. 6B). This result interpreted as a result of the activation of Doxorubicin induced drug resistance (Lovitt et al., 2018), (Chen et al., 2023).

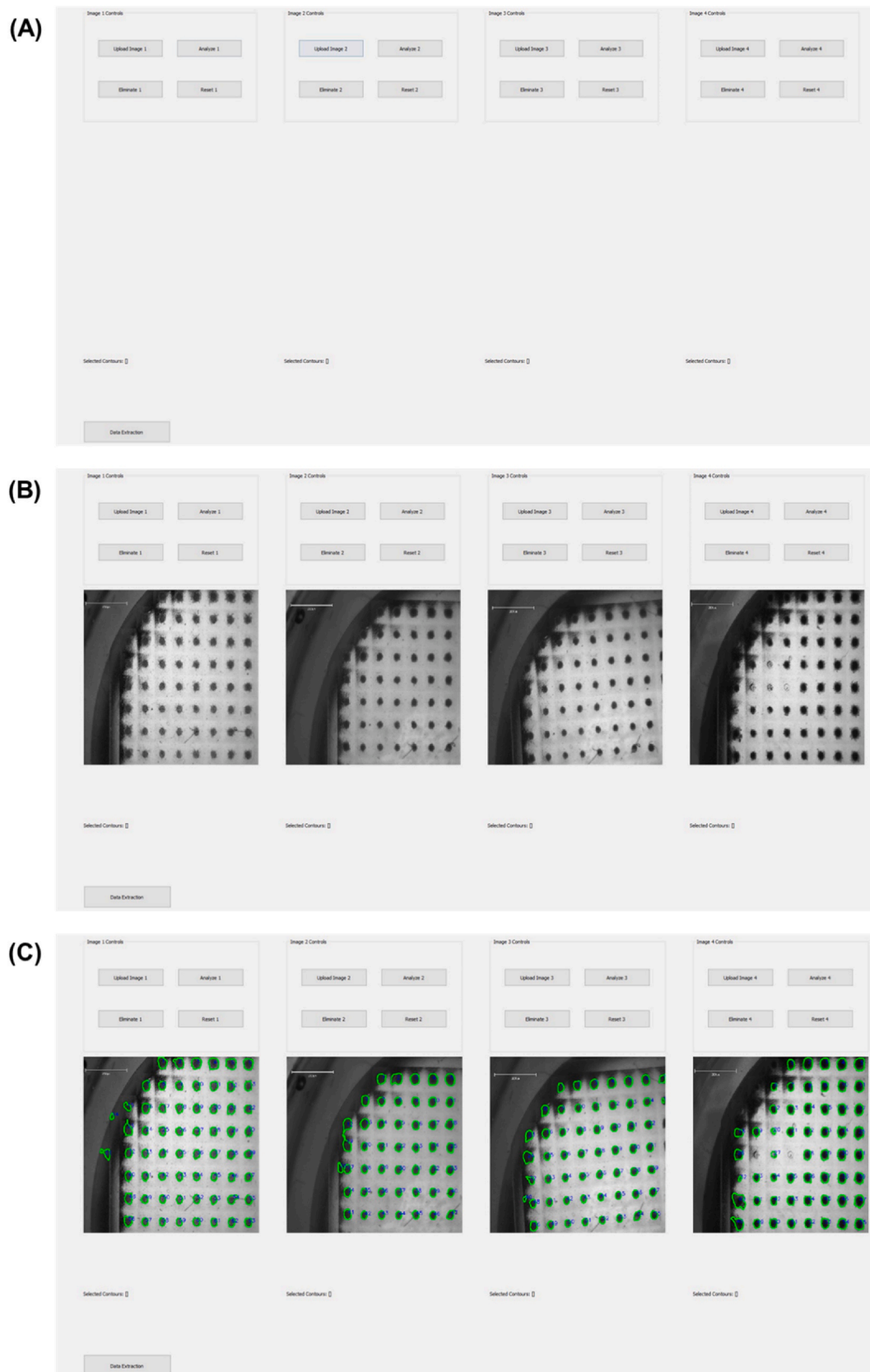


Fig. 5. (A) The default graphical user interface (GUI) when the software is first run. (B) the GUI with four uploaded images of the same microscope image taken at different days. (C) The GUI following the initiation of the spheroid detection algorithm.

Table 2
Comparison of SpAi and ImageJ based manual measurements.

Image #	# 1	# 2	# 3	#4	#5	#6	#7	#8	#9	#10
Number of Spheroids	58	37	50	60	53	60	46	50	53	61
ImageJ Mean Radius (μm)	200.18	237.10	245.79	209.21	282.77	246.60	252.57	292.83	254.30	234.20
SpAi Mean Radius (μm)	222.92	248.86	256.64	215.96	289.25	251.91	259.76	281.07	252.36	228.54
ImageJ Standard Deviation of Radius (μm)	32.40	25.58	25.81	27.29	43.32	26.49	23.06	41.15	47.20	21.75
SpAi Standard Deviation of Radius (μm)	25.13	19.82	27.36	34.14	47.53	23.83	23.09	44.60	43.85	32.85
Mean Absolute Error (μm)	28.09	12.941	15.90	8.44	10.15	6.75	7.90	21.62	16.80	22.68
Root Mean Square Error (μm)	34.23	17.91	21.54	14.83	14.80	8.75	10.79	26.78	24.28	27.56
Mean Absolute Percentage Error (%)	14.63	5.90	6.47	3.92	3.51	2.89	3.19	7.47	6.32	9.76

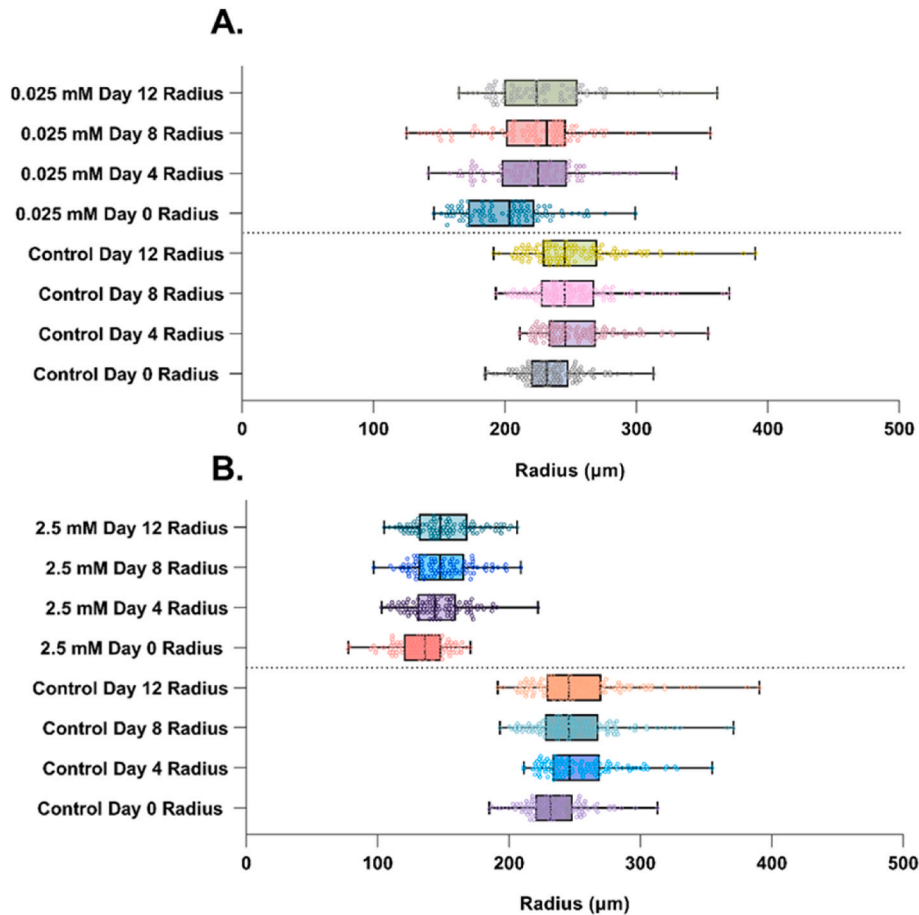


Fig. 6. (A) MCF-7 Spheroids induced with 0.025 mM Doxorubicin, (B) MCF-7 Spheroids induced with 2.5 mM Doxorubicin, size measurements by SpAi.

SpAi GUI can also be used for multiple drug concentrations and cell types in the future as an alternative to the existing methodologies.

4. Conclusion

Accurate measurement and analysis of spheroid size are crucial elements in high-throughput drug screening and cancer research. Conventional techniques for analyzing spheroids, such as human measurement and automatic segmentation using programs like ImageJ, are frequently slow and inefficient, especially when dealing with big datasets. Moreover, commercially accessible spheroid culture systems can be costly and may lack the adaptability required for tailored experimental configurations. This study introduces and verifies SpAi, a full workflow that combines 3D printing technology, PDMS microwell creation, and advanced image analysis utilizing U-Net architecture. The SpAi system effectively overcame the constraints of traditional approaches by offering a streamlined and precise way for generating spheroids and evaluating their reactions to pharmacological therapies.

The utilization of SpAi showcased various benefits: the implementation of 3D-printed PDMS molds facilitated the creation of superior spheroids, while the image analysis based on U-Net algorithm yielded accurate measurements of spheroid dimensions with minimal human involvement. The SpAi GUI enhanced the process by enabling users to analyze many photos concurrently and retrieve pertinent data with efficiency. SpAi would hold potential to provide notable advances in the sphere of spheroid-based drug screening in the future. It provides an affordable, adaptable, and efficient alternative for producing and analyzing spheroids. By incorporating machine learning and image analysis into the workflow, the accuracy and speed of data processing are improved, making SpAi a beneficial tool for both researchers and pharmaceutical businesses.

In our work, U-Net trained with a set of experimental data was obtained from a complete spheroid generation workflow. Our workflow includes an imaging strategy by dividing spheroid microwell system into 4-parts and using bright field mode to capture microscopy images. Due to that, SpAi will be optimum performing under the conditions of SpAi

workflow provides. Including more data from commercial spheroid growing techniques and microscopy methods (e.g., fluorescence microscopy) will increase application range. Considering the nature of neural networks which are highly dependent of the initial dataset for training, SpAi could be considered as a primer which will grow in the future as users interact with the workflow and adopt itself to the any experimental condition that SpAi deployed before. Common imaging artifacts including blurring, noise, and uneven illumination could also affect the performance of U-Net; yet, providing more precise data points to the training data will also resolve this challenge. Spheroid size, shape, growth stage and integrity could also affect the prediction quality of U-Net. It should be stated that in our study, high resolution microscopy images (starting from 1920×1080 pixels) used to train U-Net. Due to that, our GUI may not work as better as this quality images in low resolution images (Mizusawa et al., 2021).

In our proof-of-concept study, SpAi was successfully employed to assess the anti-cancer effect of Doxorubicin on tumor spheroids, showing a response that matched literature findings. This demonstrates the potential of SpAi to assist in evaluating drug effects on spheroids in a high-throughput manner. However, there is still some room for expansion and improvement. To broaden SpAi's applicability, the dataset used for training the U-Net model could be expanded to include spheroids from diverse cell lines, imaging conditions, and experimental variables. Additionally, SpAi's framework could be integrated with advanced imaging techniques, such as fluorescence and confocal microscopy. Fluorescence microscopy would enable the detection of specific cellular markers, while confocal microscopy could provide high-resolution, three-dimensional images, allowing for more precise morphological assessments of spheroids. These integrations would offer additional insights into spheroid structure and behavior, further improving the system's utility in complex studies involving specific cell types and dynamic cellular interactions. By incorporating these imaging modalities, SpAi could not only measure size, but also analyze internal distributions of molecular markers, live-cell imaging, and cellular responses to treatments. We are exploring these possibilities for future versions of SpAi to make it a more versatile and comprehensive tool for cancer research and drug screening.

As could be challenging for other machine learning tools and even for human experts, PDMS multi-microwell system and U-Net algorithm incorporated in SpAi could face with potential challenges. In spheroid growth, irregularities in spheroid shape, varied opacity and density across regions, changes in cell density and growth phase could change the measurements, as well as the physical properties of PDMS mold including auto-fluorescence, light scattering effects, changing surface roughness and depth variations on microwells between production periods. Imaging conditions also have a significant impact on spheroid analysis since focus variations across the field of view, uneven illumination of the mold, scattered light from the neighboring wells, depth of field limitations and magnification distortions can potentially interfere with the overall experimental results. All the aforementioned inconsistencies could also be affecting training data to create training data bias, which is a critical consideration in training phase of U-Net based image analysis algorithms. Although production defects such as multi-spheroid formation in single well, debris or bubble formation, edge growth or escaping from the wells could also affect the results, these are not a concern in SpAi analysis since those errors could be manually curated. As earlier mentioned, all the inconsistencies for both training data and experimental results could be resolved by including high quality and increased number of training datasets provided by the users for special intend with SpAi. One of the user-friendly properties of SpAi comes from its ability to generate a .xlsx file which composed of detected spheroids specific number and measured diameter. This will help users to generate faster downstream applications and automate their experimental analysis scheme if needed.

By incorporating these imaging modalities, SpAi could not only measure size but also analyze internal distributions of molecular

markers, live-cell imaging, and cellular responses to treatments. We are exploring these possibilities for future versions of SpAi to make it a more versatile and comprehensive tool for cancer research and drug screening. In order to make SpAi compatible with images containing spheroids from different cell lines or ones imaged using differing setups, a much more expansive dataset can be created with which SpAi's U-Net can be trained. Furthermore, emerging avenues such as transfer learning, where networks pre-trained on existing datasets can reduce the training time for new algorithms, can be explored. This approach will not only improve efficiency, but also extend SpAi's functionality to new areas of research. SpAi is intended to show the advantages of incorporating machine learning into spheroid based screening experiments. With experts on work for filtering through complex images, the false positive and negative rates could be further reduced when making their own dataset. Further possible future advancements may involve enhancing the system's functionalities to fit diverse cell kinds and experimental circumstances, potentially establishing SpAi as a universally utilized tool in global drug discovery and cancer research.

CRedit authorship contribution statement

Nedim Haciosmanoğlu: Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Murat Alp Güngen:** Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Eylul Gulsen Yilmaz:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Emre Ece:** Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Alphan Uzun:** Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Arda Taşcan:** Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Burak M. Görmüş:** Methodology, Conceptualization. **Ismail Eş:** Writing – original draft, Methodology, Conceptualization. **Fatih İnci:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biosx.2025.100588>.

Data availability

Data will be made available on request.

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