

Multiparametric Tumor Organoid Drug Screening Using Widefield Live-Cell Imaging for Bulk and Single-Organoid Analysis

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Abstract

Patient-derived tumor organoids (PDTOs) hold great promise for preclinical and translational research and predicting the patient therapy response from *ex vivo* drug screenings. However, current adenosine triphosphate (ATP)-based drug screening assays do not capture the complexity of a drug response (cytostatic or cytotoxic) and intratumor heterogeneity that has been shown to be retained in PDTOs due to a bulk readout. Live-cell imaging is a powerful tool to overcome this issue and visualize drug responses more in-depth. However, image analysis software is often not adapted to the three-dimensionality of PDTOs, requires fluorescent viability dyes, or is not compatible with a 384-well microplate format. This paper describes a semi-automated methodology to seed, treat, and image PDTOs in a high-throughput, 384-well format using conventional, widefield, live-cell imaging systems. In addition, we developed viability marker-free image analysis software to quantify growth rate-based drug response metrics that improve reproducibility and correct growth rate variations between different PDTO lines. Using the normalized drug response metric, which scores drug response based on the growth rate normalized to a positive and negative control condition, and a fluorescent cell death dye, cytotoxic and cytostatic drug responses can be easily distinguished, profoundly improving the classification of responders and non-responders. In addition, drug-response heterogeneity can be quantified from single-organoid drug response analysis to identify potential, resistant clones. Ultimately, this method aims to improve the prediction of clinical therapy response by capturing a multiparametric drug response signature, which includes kinetic growth arrest and cell death quantification.

Introduction

In recent years, *in vitro* cancer drug discovery, drug screening, and fundamental research have been transitioning from the use of traditional two-dimensional (2D) cancer models with immortalized cell lines to more physiologically relevant three-dimensional (3D) cancer models. This has spurred the adoption of tumor spheroids with established cancer cell lines, which recreate more complex cell-to-cell interactions and structures present in solid tumors. Currently, patient-derived tumor organoids (PDTOs) are the most advanced and physiologically relevant 3D cancer model available for *in vitro* cancer research, as they provide additional advantages over tumor spheroids, namely the heterogeneity found in cancer patients¹. PDTOs are established from tumor tissue originating from cancer patients, and therefore retain both the tumor phenotype and genotype. As such, PDTOs are becoming invaluable for fundamental and translational cancer research and have the potential to greatly improve precision oncology².

Despite their promising potential, these sophisticated 3D *in vitro* cancer models are often underutilized due to a lack of advanced analysis methods. The most commonly used assay determines the number of viable cells in the PDTO *via* the quantification of intracellular ATP³. These assays are normally single-timepoint, bulk analyses, thus overlooking critical time-dependent responses and neglecting clonal responses. Specifically, the ability to monitor the growth of PDTOs (growth rate) and their response to specific therapies is of high interest^{4,5}. The normalized drug response (NDR), which scores drug response based on the growth rate normalized to a positive (ctrl+) and negative control (ctrl-) condition, has also recently been reported to be a crucial metric for evaluating cancer drug sensitivity with cell-based

screening, though this was predominantly done for 2D cell lines⁶. Therefore, more sophisticated analysis methods are needed to fully take advantage of these more clinically representative and complex 3D cancer models. Microscopy is considered a powerful approach to study the complexity of these organoid models⁷.

This paper describes a method for monitoring kinetic drug responses in 3D cancer models, using conventional widefield microscopes and live-cell imaging systems. Adaptations were made to the protocol described by Driehuis et al.⁴ to be compatible with automation using a pipetting robot, digital drug dispenser, and live-cell imaging system to increase reproducibility and reduce the number of 'hands-on' hours of labor. This method allows for medium- to high-throughput drug screening of both tumor spheroids with established cancer cell lines (see **Supplementary Table S1** for tested cell lines), as well as the PDTOs, in a 384-well microplate and multi-organoid format. By using a convolutional network machine learning process, automated identification and tracking of individual tumor spheroids or PDTOs could be performed solely from brightfield imaging and without the use of fluorescent live-cell labeling dyes⁸. This is highly advantageous, as most identification with brightfield imaging requires manual annotation (which is laborious and time-consuming) or requires the addition of fluorescent dyes, which can confound drug responses related to phototoxicity-induced oxidative stress⁹.

The resulting image analysis software developed in-house extends the functionality of conventional live-cell imaging systems, as 3D image analysis modules are either not available, platform-restricted, or not compatible with 384-

well microplates and whole-well imaging. In addition, these modules are often highly priced and offer limited bulk organoid readouts. Therefore, this method is highly relevant for scientists who have access to widely available live-cell imaging systems and aim to extract more information about a drug response compared to the gold-standard but rudimentary ATP-based assay. With the addition of specific cell death indicators, cytostatic drug responses can be distinguished from cytotoxic responses, thus providing further insight into mechanistic drug actions currently unattainable from single-timepoint analysis. Finally, live-cell imaging allows for individual organoid tracking to obtain single organoid drug response metrics to capture response heterogeneity and identify potential resistant subclones.

The goal of this method and the associated image analysis software is to implement low-cost automation in organoid drug screening to limit user intervention and reduce variability in handling, image analysis, and data analysis. To make this software available to researchers, it is microscope- and platform-agnostic, and a cloud-based application is made available. Thus, by supporting conventional live-cell imaging systems, we also aim to improve their functionality for 3D culturing applications and analysis.

Protocol

Human pancreatic ductal adenocarcinoma (PDAC) patient-derived organoids were used. Tissue resection fragments were obtained from patients undergoing curative surgery at the Antwerp University Hospital. Written informed consent was obtained from all patients, and the study was approved by the UZA Ethical Committee (ref. 14/47/480). Details related to all materials, reagents, equipment, and software used in this protocol are provided in the **Table of Materials**. An overview of the workflow is presented in **Figure 1**. Example

data is provided in the supplementary material to reproduce the protocol.

1. Day 0: Preparation of 2- or 3-day old organoids

1. Preheat the microplates at 37 °C overnight and thaw the extracellular matrix (ECM) at 4 °C.
2. Prepare full PDAC organoid culture medium: supplement ADF+++ (Advanced DMEM/F12, 1% glutamine supplement, 1% HEPES, and 1% penicillin/streptomycin) with 0.5 nM WNT surrogate-Fc-Fusion protein, 4% Noggin-Fc Fusion Protein conditioned medium, 4% Rps3-Fc Fusion Protein conditioned medium, 1x B27, 1 mM N-acetyl cysteine (NAC), 5 mM nicotinamide, 500 nM A83-01, 100 ng/mL FGF10, and 10 nM Gastrin.
3. Establish the PDOs according to the method of choice. **NOTE:** A detailed protocol is provided by Driehuis et al., which describes the conventional method to establish, culture, and passage PDOs in ECM domes⁴.
4. Enzymatically dissociate the organoids in ECM domes.
 1. Aspirate the medium and wash 1x with phosphate-buffered saline (PBS). Add dissociation enzyme (e.g., 2 mL in a 6-well microplate) and pipette up and down 10x with a 1 mL pipette to mechanically dissociate the organoids and ECM domes.
 2. Incubate for 10 min at 37 °C, pipette up and down, and check whether the organoids are dissociated to single cells. Repeat this step if necessary.
 3. Collect the cell suspension in a 15 mL tube, add ADF+++ to a volume of 10 mL, centrifuge for 5 min at 450 × g at room temperature, and aspirate the supernatant with a Pasteur pipette and suction pump.

4. Resuspend the pellet in 100-200 μL of full medium depending on the size of the pellet and count the number of cells using the method of choice. For example: mix 10 μL of the cell suspension + 10 μL of Trypan Blue and count with an automated cell counter.
5. Plate single cells in ECM domes.
 1. Dilute the cell suspension and add 2/3 ECM according to **Table 1**. Pipette up to ten 20 μL droplets per well in a preheated 6-well plate. Invert the plate and incubate for 30 min at 37 °C.
 2. Overlay with full medium supplemented with 10 μM Y-27632 and incubate for 2-3 days in an incubator.

NOTE: Ten domes containing 75,000 cells each are usually enough to fill one 384-well microplate at a concentration of 200 organoids/well, excluding the wells at the edge.

2. Days 2 - 3: Harvest and seed 2- or 3-day old organoids

1. Collect intact organoids from the ECM domes.

NOTE: Organoids tend to stick to plastic surfaces (e.g., tubes, pipette tips). To avoid this, plasticware can be prerinsed with a 0.1% bovine serum albumin (BSA)/PBS solution.

 1. Aspirate the medium and wash 1x with PBS. Add 1-2 mL of cold (4 °C) organoid harvesting solution to a 6-well plate depending on the number of ECM domes and incubate on ice on a shaking platform for 10 min.
 2. Pipette up and down with a 1 mL pipette to dissociate the ECM domes, incubate for an additional 10 min on ice, and visually check under a microscope whether the ECM is dissociated.

3. Optional: if a more uniform size distribution is preferred, filter the suspension through a 70 μm cell strainer before centrifugation.
4. Collect the organoids in a 15 mL tube precoated with 0.1% BSA/PBS, add ADF+++ up to 10 mL, and centrifuge for 5 min at 200 $\times g$ at 4 °C. Aspirate the supernatant and resuspend the pellet in up to 1,000 μL of full PDAC organoid medium depending on the size of the pellet to obtain a concentration of >6,000 organoids/mL.
5. Count the organoids using any counting method of choice, preferably an image-based one.
2. Seed the organoids.

NOTE: See the **Table of Materials** for the minimum volume/well for two different types of 384-well microplates used in this protocol.

1. Precool all plasticware at -20 °C or on ice for at least 20 min before use to avoid solidification of the ECM.
2. Prepare the seeding solution from the 1 mL of organoid stock solution (step 2.1.6) using full medium to seed ~200 organoids per well in 50 μL , the minimum volume used to fill a well. Use **Supplementary File 1** to calculate the amount of organoid seeding solution. Add a residual volume of 1,500 μL when using a 25 mL reservoir and multichannel pipette or pipetting robot.
3. Seed the organoids using a pipetting robot.

NOTE: Both the seeding solution and microplate need to be cooled at 4 °C during pipetting to avoid solidification of the ECM. Therefore, a 25 mL reservoir and microplate holder were 3D printed to be used in combination with the pipetting robot that can hold cooling elements listed in

the **Table of Materials**. STL files for 3D printing the custom labware (**Supplementary File 2** and **Supplementary File 3**) and custom labware JSON files for the pipetting robot (**Supplementary File 4** and **Supplementary File 5**) are provided.

1. Design the dispensing protocol using the online Protocol Designer Tool. An example JSON file (**Supplementary File 6**) is provided, in which the custom labware is already loaded and an eight-channel p300 (Gen2) pipette with corresponding pipette tips is used.
2. Open the pipetting robot control app, select **protocols**, click on **Import**, and drag and drop **Supplementary File 6** into the designated field.
3. Select the imported protocol and place all the labware, including cooling elements and plasticware, in the decks according to the layout shown in the **Deck Setup** field. Use the left slot for the 25 mL reservoir and cooling element as shown in **Supplementary File 7**.
4. Click on **Run Protocol** and **Proceed to Setup**. Open the **Labware Setup** tab, click on **Run Labware Position Check**, and follow the instructions to calibrate the pipetting robot to the new hardware.

NOTE: Labware offset data can be stored for later, but it is recommended to run the labware position check before each run.

5. Fill the 25 mL reservoir (placed on top of the cooling element) with the cooled organoid seeding solution and click on **Start Run**.

NOTE: The top and bottom wells also become filled with organoid suspension solution due to the use of the eight-channel pipette.

6. Centrifuge the microplate for 1 min at 100 × g at 4 °C.
7. Incubate at 37 °C for at least 30 min.
8. Fill the outer blank wells with at least 50 µL of H₂O to avoid evaporation.
9. Incubate at 37 °C overnight to remove any bubbles in the well that can interfere with the image analysis.

3. Day 4: Drug treatment and reagent dispensing with digital drug dispenser

1. Create the drug dispensing protocol using the digital drug dispenser control software.
 1. Hover over Plate 1 above the plate layout, select **edit plate attributes**, and fill in **plate type: 384 well**, **additional volume (µL): 50**, and **DMSO limit (%): 1**.
 2. Add fluids by clicking on the + button next to **Fluids**. Double-click on **the newly created fluid** and name it; select **class** (DMSO-based or aqueous+Tween 20) and **concentration**.

NOTE: All drugs and reagents must be dissolved in 100% DMSO or 0.3% Tween-20. A 1-10 mM stock solution can be used, taking into account a maximum DMSO concentration of <1%. **Table 2** provides examples of the required dilutions for common fluorescent reagents and therapies.
3. Plate layout
 1. For drug titration, select wells and click on **Titration**. For **fluid**, select the **drug of interest**,

choose the **highest concentration** (e.g., 2,000 nM), and the **lowest concentration** (e.g., 10 nM); for **replicates**, choose a **minimum of 2**, and choose the desired **titration pattern**.

NOTE: The titration pattern will depend on many factors, including how much compound is to be fit in a single plate, whether the wells are to be randomized, and the number of replicates and controls.

2. For the **positive control**, select three wells, click on **Set Value**, and fill in 2 μ M staurosporine from 10 mM stock in DMSO, which will induce maximum cell death.

3. For Cytotox Green, select all the used wells, click on **Set Value**, and enter 60 nM/well.

NOTE: The Cytotox Green fluorescent staining indicates cells that have died, and therefore will not interfere with drug response monitoring. Here, no fluorescent marker for live cells is required.

4. For the negative control and DMSO normalization, select all the wells with an additional four wells for the vehicle control, right-click, select **normalization**, select **normalize fluid class: DMSO-based**, and normalize to the highest class volume to obtain an equal DMSO concentration in each well.

NOTE: DMSO concentrations should be <1%. An example TDD drug titration file (**Supplementary File 8**) is provided.

5. Click on the **arrow** under **Run** in the top left corner, select **Always Simulate**, and click on

Simulate to identify any errors and obtain the volumes of each drug to be prepared.

NOTE: To overcome a warning when the initial dispensing volume is too low, "Dispense Warning well of 30 nL or greater is recommended for each fluid on each plate," select two wells on the edge that are filled with water, select **Set Value**, and enter **10 μ M** of the drug for which the warning occurs. This primes the drug cartridge with a volume higher than 30 nL. These same wells can be used to prime the DMSO cartridge by setting a **normalize to % of total volume** value (e.g., 0.5%).

2. Uncheck **Always Simulate** under the **Run** button; click on **Run** to start the drug dispensing protocol and follow the instructions.

3. Apply the sealing membrane to the microplate to prevent evaporation.

4. Incubate the Cytotox Green dye 1-2 h at 37 °C in the incubator and proceed to step 4.

4. Acquire images with the live-cell imager

NOTE: For the growth rate and NDR, a scan at timepoint 0 (T0 = start treatment) must be acquired 1-2 h after adding Cytotox Green.

1. Open the live-cell imager Control software, select **Method Editor New**, go to **file > import**, and select the **example method XML file (Supplementary File 9)**. Alternatively, create a new file and select **Plate: (CORE384fb_OpticalImaging) - Corning 384 Flat Black (Corning #4588), No lid and No humidity cassette; Application: Images-only; Objective: 4x; Pattern: Central; Check channels Brightfield and**

Green (Led intensity (%)) = 40; Exposure Time (ms) = 200).

NOTE: The green channel settings work well for a concentration of 60 nM Cytotox Green. The **Live viewer** option can be used to adjust the focus offset and/or the led settings in real time.

2. Click on **Start** to initiate scanning at T0.
3. Repeat the scan every 24 h for up to 5 days using the same method. Alternatively, to run the timelapse measurement automatically, adjust the method in the live-cell imager Control software to a kinetic experiment by clicking and dragging the **Kinetic Loop** tab into the **method** field. Similarly, the **Temperature** and **Gas** tabs have to be dragged into the **method** field to set the system to 37 °C and **5% CO₂** to ensure the correct conditions within the live-cell imager during the experiment.

5. Image and data analysis

1. Merging and compressing data

1. The live-cell imager Control software generates a folder for each scan at each timepoint. Create a new folder, copy the individual experiment folders into this new parent folder, and add **_0h**, **_24h**, **_48h**, **_72h**, **_96h**, and **_120h** to the corresponding experiment folder names.
2. Prepare an XLSX plate map from the digital drug dispenser Control software by right-clicking on the **plate map** layout from the drug dispensing protocol and copy all the wells; paste the data in an XLSX file. Remove Cytotox Green and staurosporine data and add a matrix for Cell Line and Replicate. Enter

ctrl- and **ctrl+**. See **Supplementary File 10** for an example plate map.

3. Open the **Data Compression Tool**, click on **Browse**, select the **parent folder**, and click on **Run** to initiate image data compression. All TIFF image files for the different timepoints are compressed into a single HDF5 for each well in a new datasets folder within the parental folder.

2. Image analysis

1. Go to the image analysis webapp platform, log in, and click on **Add New Project** in the **Home** tab. Enter the **project name**, **continue**, select **Add New Experiment**, and upload the datasets folder containing the HDF5 files.
2. After uploading, go to the **project** and **experiment folder** and click on **Upload Platemap** for additional functionalities. Click on **Run Analysis**, **select Multi-Organoid analysis**, **Default Parameters**, and click on **Analyze** to initiate image analysis.
3. Click on **Download Results** to download the raw data tables which contain the measurements for each well (e.g., total brightfield area, total fluorescence green area, etc.) and the segmented images/videos to confirm the accuracy of the analysis and further data processing.

3. Growth rate-based drug response metrics and normalized drug response

1. Select the Raw_NDR.xlsx file from the **results** folder (plate map required) (**Supplementary File 11**) and load this into the Official_NDR_7point R-script (**Supplementary File 12**) to automatically generate GR (normalized to ctrl-) and NDR (normalized to ctrl- and ctrl+) value tables (**Supplementary File**

13, Supplementary File 14, Supplementary File 15, and Supplementary File 16). GR and NDR values are calculated from the parameter as shown in equation (1) using the R-script (**Supplementary File 12**).

$$\text{Total Survival Area} = \text{Total Brightfield Area} - \text{Total Green Area} \quad (1)$$

Where $0 < \text{NDR} < 1$ = cytostatic effect (growth arrest), and $\text{NDR} < 0$ = cytotoxic response (cell death).

NOTE: The R-script was adapted from Gupta et al.⁶.

2. From the clonal_data.xlsx table, retrieve single-organoid response data and plot them as a bubble plot.
3. Use the Z-factor¹⁰ to assess the drug screen quality of a run (see equation (2)). Discard an experiment with a Z-factor < 0.5 .

$$Z = 1 - \frac{3 \times SD(\text{negative control}) + 3 \times SD(\text{positive control})}{\text{average}(\text{negative control}) - \text{average}(\text{positive control})}$$

(2)

Representative Results

The automated pipetting protocol ensures an even distribution of PDAC_060 PDTOs in all the columns of the 384-well microplate (**Figure 2A**). As expected, a variation in the number and mean area of PDTOs was observed between the wells (**Figure 2A,B**). The total survival area (total brightfield area - total green area) combines the label-free organoid segmentation with the fluorescence-based cell death signal and is, in our experience, the most robust parameter to study drug responses over time (**Figure 2C**)⁸. To account for variations in cell seeding and organoid size, growth rate-based metrics should be used to reduce variations between replicates, as shown by the reduced error bars in **Figure 2D** versus **Figure 2C**, and a higher Z-factor

indicating a strongly improved drug screen quality (**Figure 2E**).

The NDR dose-response curve (**Figure 2G**), normalized to ctrl- and ctrl+, is clearly superior to the GR dose-response curve (**Figure 2F**), normalized to ctrl-, as it increases the separation of the drug response curves and more accurately represents cytotoxic drug responses. An example of the associated images for ctrl-, ctrl+, and 400 nM gemcitabine/80 nM paclitaxel-treated PDTO is shown in **Figure 3**. An interesting observation is that the cytotoxic effect of gemcitabine was dominant in the combination therapy as no added value of paclitaxel was observed.

Next, two additional PDTO lines, PDAC_052 and PDAC_087, were used. A clear difference in growth rate between these lines was observed (**Figure 4A**), which supports the use of GR metrics. Again, NDR dose-response curves (**Figure 4C**) resulted in an increased dynamic range and separation between the three different patients compared to the GR curves (**Figure 4B**). Furthermore, the protocol allows for the determination of NDR over time and shows that PDAC_052 and PDAC_060 had a very similar cytostatic drug response to a low dose of gem-pac (**Figure 4D**), while a clear differential cytostatic versus cytotoxic response could be observed for the middle (**Figure 4E**) and high doses (**Figure 4F**) of gem-pac. These drug responses were consistent with the clinical responses observed in the patients (**Figure 4G**).

Finally, a major benefit of the approach and software is that single-organoid drug responses can be quantified to study response heterogeneity and identify potentially resistant subclones. **Figure 5** provides a clear overview of the clonal dynamics of the different patients and shows that PDAC-087 had the most resistant subclones following treatment, which is consistent with the aggressive and highly resistant disease

observed in the patient. Interestingly, this patient was also the least sensitive to the ctrl+ staurosporin.

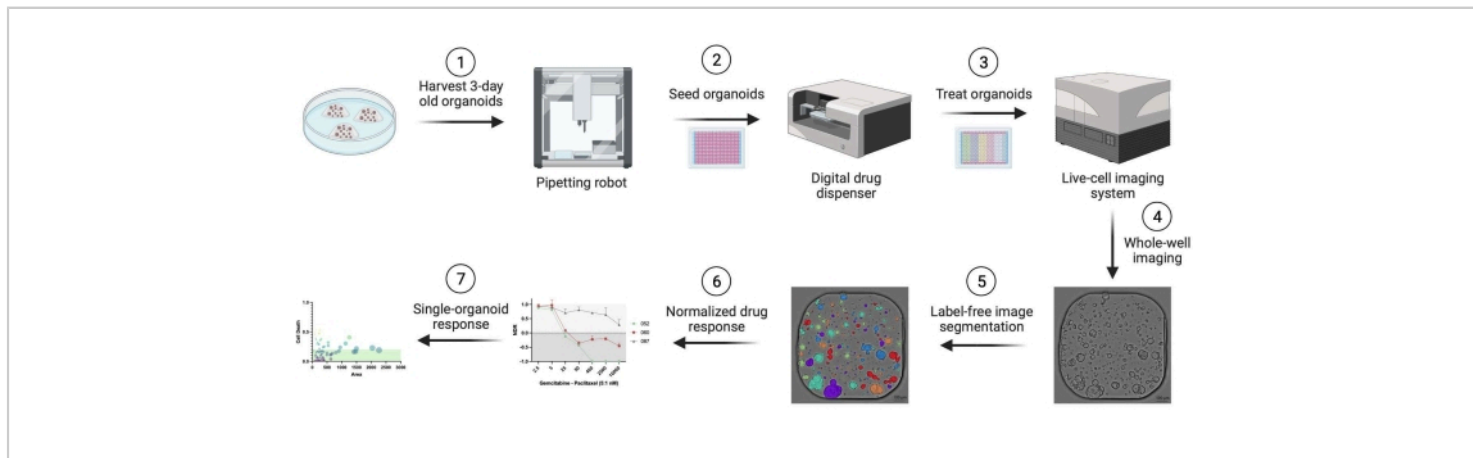


Figure 1: Workflow overview. Please [click here](#) to view a larger version of this figure.

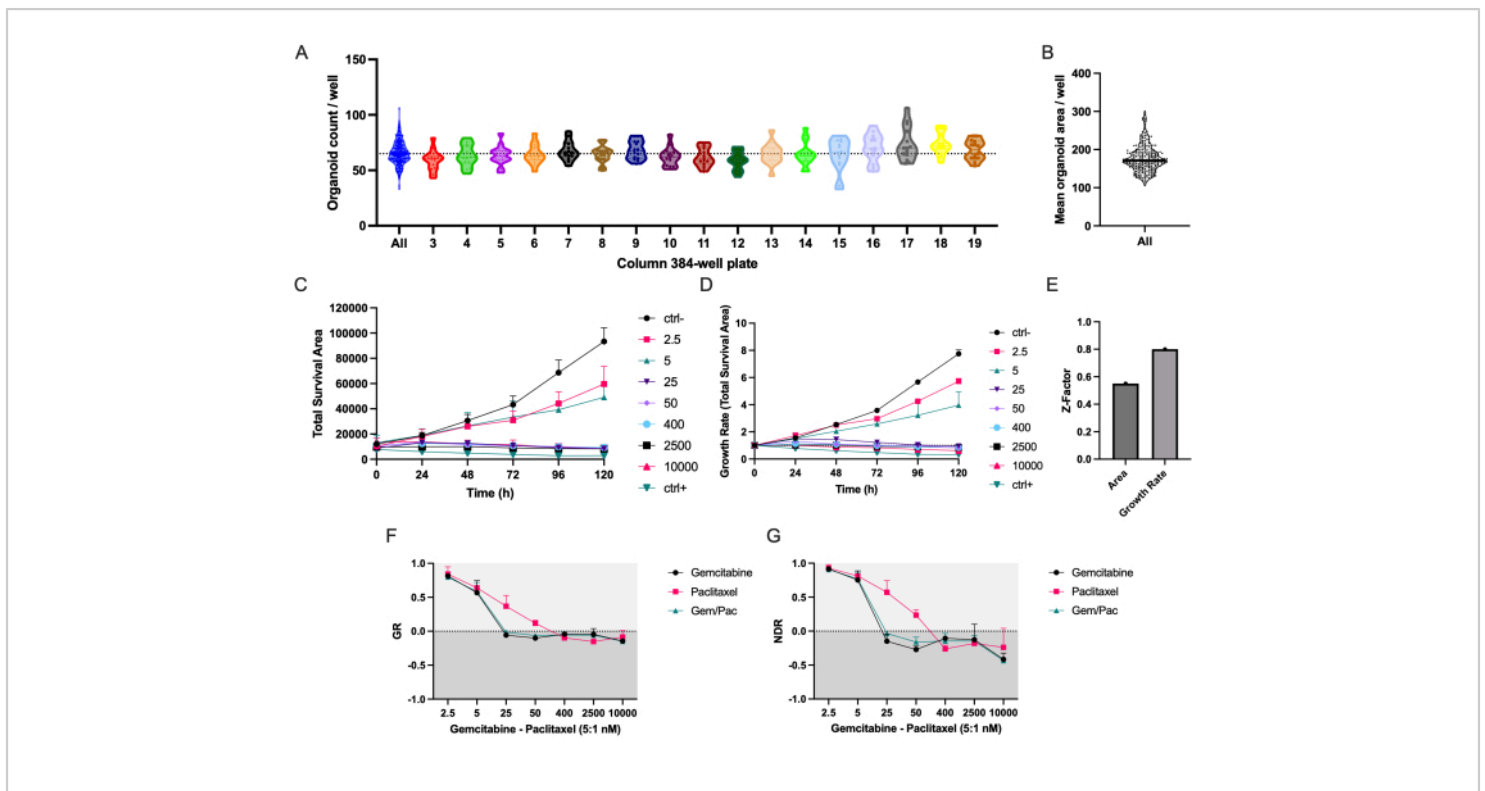


Figure 2: Seeding accuracy and drug response metrics. (A) Organoid counts/well of PDAC_060 PDTOs seeded in a 384-well microplate using the pipetting robot. Each dot represents the count in a single well and plots are separated by the 384-well microplate columns. (B) Mean PDTO area/well. (C) Total survival area (total brightfield area - total green area) and (D) growth rate (total survival area normalized to T0 = 1) of PDAC_060 PDTOs treated with a 5:1 ratio of gemcitabine/paclitaxel. (E) Z-factor as a metric for assay quality. (F) Growth rate-dose response curve normalized to ctrl- and (G) normalized drug response curve normalized to ctrl- and ctrl+. Error bars indicate mean \pm SD of two wells. Abbreviations: PDAC = pancreatic ductal adenocarcinoma; PDTO = patient-derived tumor organoid; GR = growth rate; NDR = normalized drug response. [Please click here to view a larger version of this figure.](#)

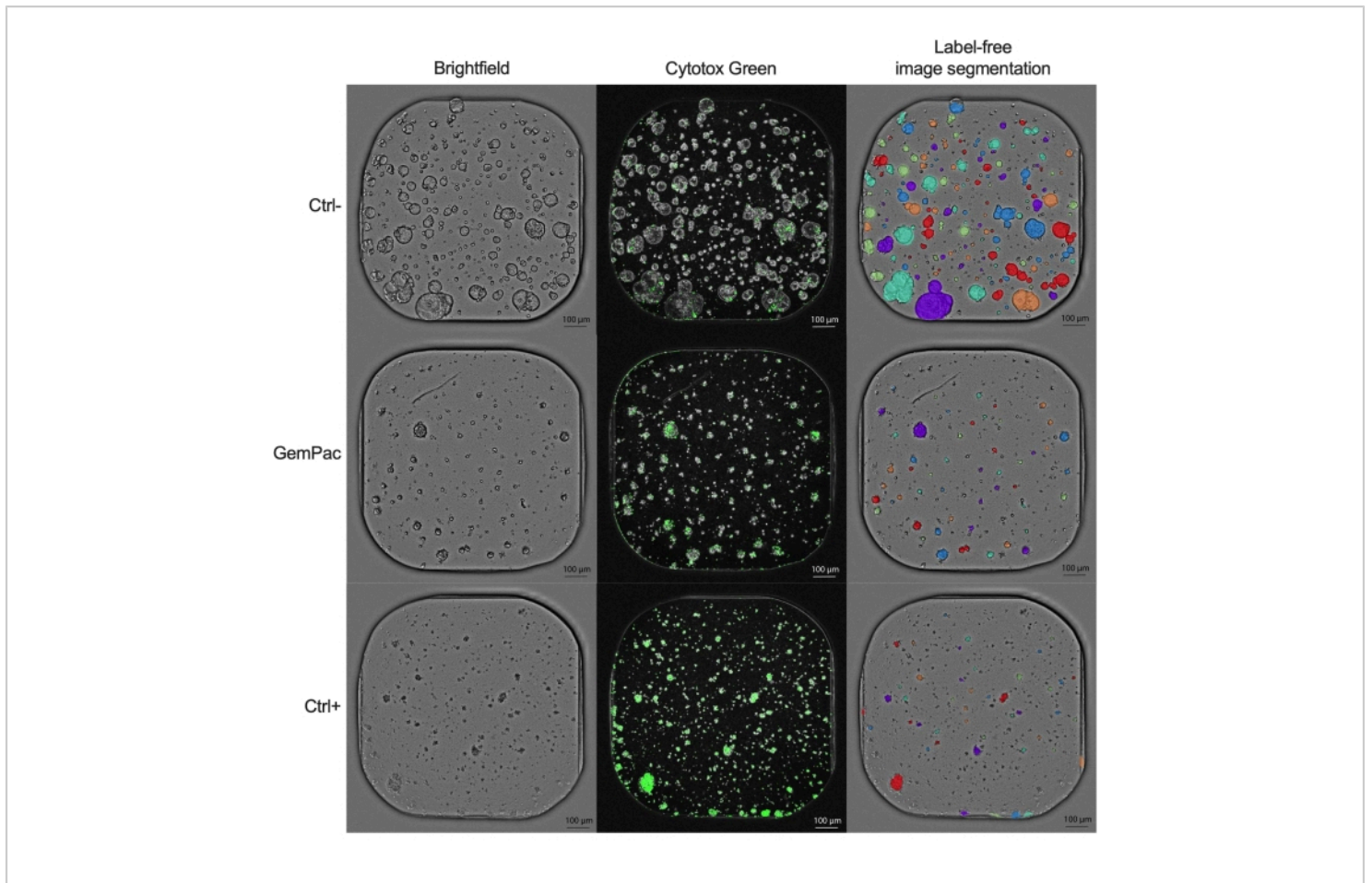


Figure 3: Example images. Representative images of PDAC_060 PDTO treated with vehicle (ctrl-), 400 nM gemcitabine/80 nM paclitaxel, and 2 μ M staurosporin (ctrl+). The left column shows brightfield images, the middle column shows the Cytotox Green fluorescent signal, and the right column shows the label-free annotated brightfield images using the organoid analysis module. Scale bars = 100 μ m. Abbreviations: PDAC = pancreatic ductal adenocarcinoma; PDTO = patient-derived tumor organoid; GemPac = gemcitabine/paclitaxel. [Please click here to view a larger version of this figure.](#)

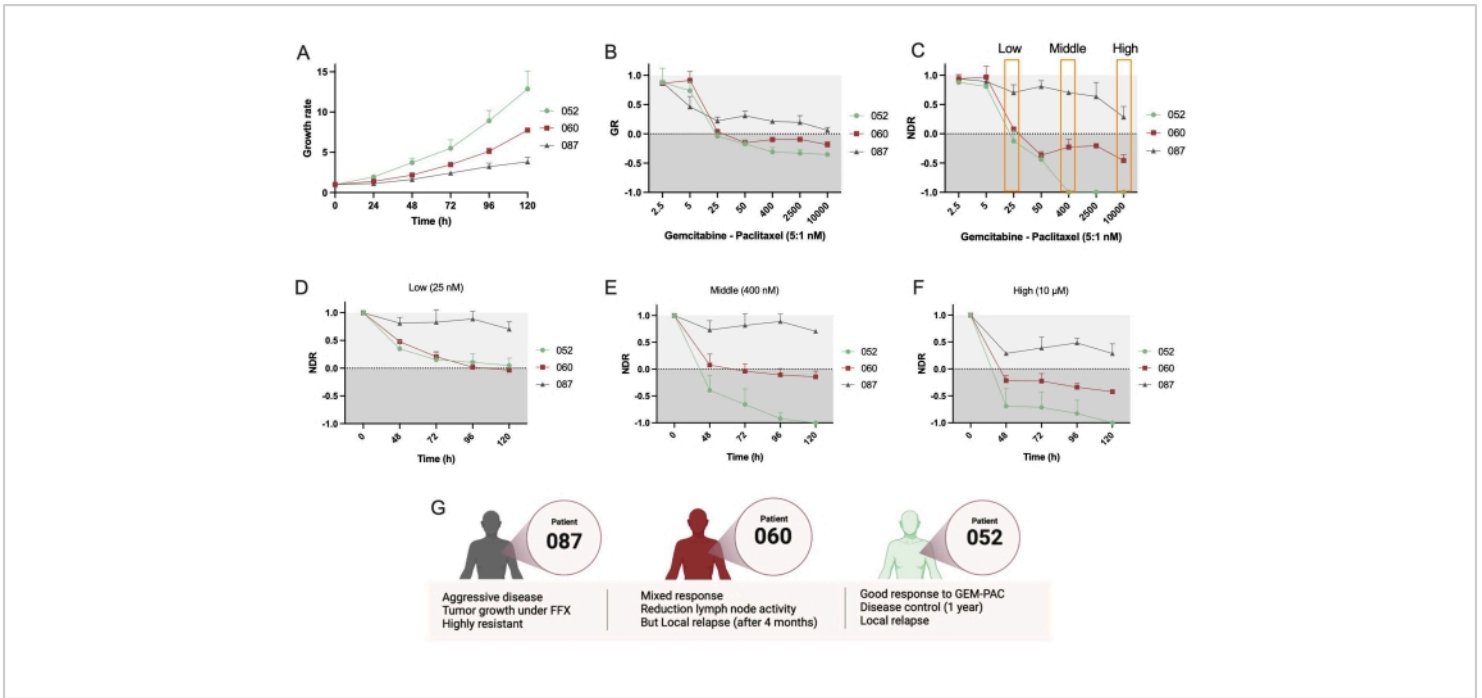


Figure 4: Comparing interpatient drug response. (A) Comparison of growth rate (based on total survival area) of PDAC_052, PDAC_060, and PDAC_087 PDTO lines. (B) Growth rate-dose response curve normalized to ctrl- and (C) normalized drug response curve normalized to ctrl- and ctrl+. Kinetic NDR of a (D) low, (E) middle, and (F) high dose of gemcitabine/paclitaxel (5:1 ratio). (G) PDAC patients' clinical characteristics. Error bars indicate mean \pm SD of two wells. Abbreviations: PDAC = pancreatic ductal adenocarcinoma; PDTO = patient-derived tumor organoid; GR = growth rate; NDR = normalized drug response; FFX = folfirinox. [Please click here to view a larger version of this figure.](#)

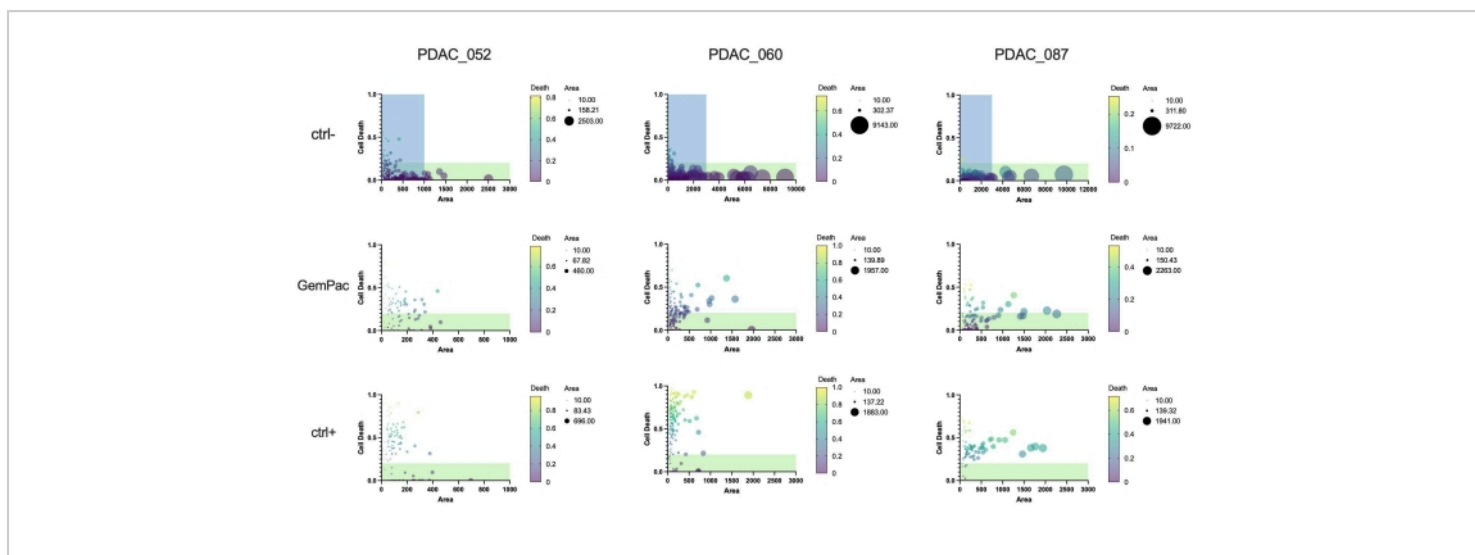


Figure 5: Single organoid metrics. Single organoid dose response based on cell death (green area/brightfield area) and area (brightfield) of PDAC_052, PDAC_060, and PDAC_087 PDTOs treated with vehicle (ctrl-), 400 nM gemcitabine/80 nM paclitaxel, and 2 μ M staurosporine (ctrl+). Green regions indicate viable organoids; blue regions indicate x-as range of GemPac and ctrl+ plots. Abbreviations: PDAC = pancreatic ductal adenocarcinoma; PDT0 = patient-derived tumor organoid; GemPac = gemcitabine/paclitaxel. [Please click here to view a larger version of this figure.](#)

Cell suspension stock	Cells/Drop	# Drops (20 μ L)	Stock (1/3)	ECM (2/3)
1.13×10^7 cells/mL	75,000	10	75 μ L	150 μ L
1.13×10^7 cells/mL	75,000	5	40 μ L	80 μ L

Table 1: Dilution for plating in ECM domes. Abbreviation: ECM = extracellular matrix.

Compound	Stock concentration	Dilution	Working concentration	Solvent	Well concentration	Comments
Cytotox Green	1 mM (DMSO)	1/10	10 μ M	DMSO	60 nM	Cell death marker
Cytotox Red	1 mM (DMSO)	1/10	10 μ M	DMSO	250 nM	Cell death marker
Caspase 3/7 Green	5 mM (DMSO)	1/2	2.5 mM	DMSO	2.5 μ M	Apoptotic marker
Hoechst	20 mM (H ₂ O)	1/200	100 μ M	0.33% Tween/PBS	50 nM	Nuclear marker
Staurosporin	10 mM (DMSO)	/	1 - 10 mM	/	2 – 5 μ M	Positive control
Gemcitabine	10 mM (DMSO)	/	1 - 10 mM	/	Titration	Chemotherapy
Paclitaxel	10 mM (DMSO)	/	1 - 10 mM	/	Titration	Chemotherapy
Cisplatin	5 mM (0.9% NaCl)	1/2	2.5 mM	0.6% Tween/PBS	Titration	Chemotherapy

Table 2: Example dilutions of frequently used drugs and fluorescent reagents. Each compound needs to be dissolved in either 100% DMSO or 0.3% Tween/PBS.

Supplementary Table S1: Overview of compatible cancer cell lines. Static: spheroids are not migratory. Merge: spheroids migrate toward each other and merge together. [Please click here to download this File.](#)

Supplementary File 1: Organoid seeding solution calculation tool. [Please click here to download this File.](#)

Supplementary File 2: STL file for 3D printing custom labware 'Microplate Holder'. [Please click here to download this File.](#)

Supplementary File 3: STL file for 3D printing custom labware '2 x 25 mL Reservoir Holder'. [Please click here to download this File.](#)

Supplementary File 4: JSON file for custom labware pipetting robot 'Microplate Holder'. [Please click here to download this File.](#)

Supplementary File 5: JSON file for custom labware pipetting robot '2 x 25 mL Reservoir Holder_WithCooler'. [Please click here to download this File.](#)

Supplementary File 6: JSON file for pipetting robot protocol 'Plating_PDO_384well_Cooled_Row2-23'.

[Please click here to download this File.](#)

Supplementary File 7: Overview of the pipetting robot desk setup. (A) Cooling elements and (B) reservoir and microplate. [Please click here to download this File.](#)

Supplementary File 8: TDD file for protocol of the digital drug dispenser. [Please click here to download this File.](#)

Supplementary File 9: XML file for protocol of the live-cell imager for brightfield and fluorescence imaging. [Please click here to download this File.](#)

Supplementary File 10: Example plate map. [Please click here to download this File.](#)

Supplementary File 11: Example input file for NDR R script. Abbreviation: NDR = normalized drug response. [Please click here to download this File.](#)

Supplementary File 12: Normalized drug response NDR R script. Abbreviation: NDR = normalized drug response. [Please click here to download this File.](#)

Supplementary File 13: Example output file of NDR R script GR values. Abbreviations: GR = growth rate; NDR = normalized drug response. [Please click here to download this File.](#)

Supplementary File 14: Example output file of NDR R script with GR values transposed. Abbreviations: GR =

growth rate; NDR = normalized drug response. [Please click here to download this File.](#)

Supplementary File 15: Example output file of NDR R script NDR values. Abbreviation: NDR = normalized drug response. [Please click here to download this File.](#)

Supplementary File 16: Example output file of NDR R script with NDR values transposed. Abbreviation: NDR = normalized drug response. [Please click here to download this File.](#)

Discussion

Medium- to high-throughput PDTO drug screening often relies on readouts that only extract a fraction of information that organoids could potentially provide. It has become increasingly clear that, in order for the rapidly evolving organoid technology to realize greater scientific and clinical potential, more advanced 3D assays, readouts, and analysis methods are critically required. Here, an advanced screening pipeline is described, which not only increases the reproducibility, but also considerably enhances the clinical translatability by incorporating an AI-driven, live-cell imaging readout. In addition to analysis software developed in-house, the use of the normalized drug response metric (NDR) is implemented, which clearly demonstrates its ability to define patient-specific differences in treatment response⁶.

The inclusion of this normalization metric will undoubtedly be of tremendous value, recalling that numerous studies aim to delineate treatment responses based on minor differences in area under the curve (AUC) or half-maximal inhibitory concentration (IC₅₀) (as most of the dose-response curves overlap/are located close to each other)^{11,12}. Growth rate

metrics have already been implemented in organoid drug screening protocols using the ATP-based assay but rely on the normalization of reference wells lysed at timepoint 0⁴. In contrast, this method allows for intrawell growth-rate normalization, which not only accounts for interpatient differences in PDTO growth rate but also interwell differences resulting from variations in seeding density and plate location-dependent effects to increase reproducibility. Furthermore, we adapted the NDR to further increase the separation of interpatient PDTO response by including a positive control for normalization^{6,8}.

Furthermore, the analysis, which is compatible with high-throughput and automation formats, can accurately detect individual organoid responses, enabling the quantification of subclonal resistance—the major driving force of tumor relapse and progression¹³. For example, although PDAC052 and PDAC060 showed a good response to the treatment *in vitro* (based on the NDR), the additional single-organoid analysis was able to detect a small (bigger population with PDAC060) population of subclones that do not respond to the treatment. Interestingly, this corresponded highly with the clinical observation, given that PDAC052 and PDAC060 had a durable response (no tumor activity detected) but eventually were both diagnosed with local tumor progression (due to the presence of resistant clones). Compared to the conventional 3D readouts (ATP-based assay and size/numbers), this advanced screening pipeline is expected to increase the predictive performance by extracting more clinically relevant information out of these 'patients-in-the-lab'. This hypothesis is now being tested by screening clinical PDTO samples in the authors' laboratory with this method to correlate *ex vivo* with *in vivo* response and clinical outcome.

To obtain more insights into the mechanisms of a drug response, conventional fluorescent live-cell imaging reagents, in addition to cytotoxicity dyes, are compatible with this method to study mechanisms of cell death. We have previously shown the compatibility of this method with the Sartorius Caspase 3/7 Green Reagent to study caspase-dependent induction of apoptosis following cisplatin treatment⁸. The compatibility with other dyes to study oxidative stress (CellROX reagents) or hypoxia (Image-iT Hypoxia reagents) remains to be tested. However, these reagents have already successfully been used in 3D *in vitro* models^{14,15}.

The image analysis software is also compatible with other plate formats or culturing methods (e.g., microcavity plates, ECM domes) if clear, in-focus images of the organoids can be captured. This is often challenging for organoids cultured in domes since they grow in different z-planes, which requires z-stacking functionality of the microscope that is not always available. Therefore, we advise the use of flat-bottom ULA 384-well microplates to ensure images of sufficient quality.

In addition, the analysis is compatible with other live-cell imaging systems, as previously shown for phase-contrast images captured with an IncuCyte ZOOM system⁸. A limitation of the Spark Cyto live-cell imaging system that was used in this manuscript is the one-plate capacity for kinetic measurements. However, the Spark Motion expansion increases its capacity to up to 40 microplates that can be screened in bulk. The compatibility of the software developed in-house will be expanded to these and other systems to offer a platform-agnostic solution, with the goal to standardize and automate image and data analysis pipelines. The web-based application will also include interactive graphing tools and

automated drug metric calculations, as shown in this paper, to reduce manual analysis time.

The label-free PDO segmentation algorithm was trained and tested on various in-house grown spheroid and PDO models with distinct morphological differences (solid, semi-solid, cystic), and can consequently detect these with high accuracy⁸. A limitation of the model is that the inclusion of cystic PDOs increased the unwanted detection of bubbles present in the well following seeding. However, overnight incubation was sufficient to remove most of these bubbles, allowing for a qualitative timepoint 0 scan. The accuracy of the organoid image segmentation and the method needs to be validated by other users, and based on their feedback, the software can be trained further to obtain a robust and automated image analysis algorithm. In addition, we aim to obtain more clinical data to correlate the *ex vivo* drug response quantified by this method to the clinical response in the patient to identify the best parameters to predict therapy response and further develop this method for functional precision cancer medicine¹⁶.

Disclosures

The authors declare no conflict of interest.

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