jove JOURNAL OF VISUALIZED EXPERIMENTS
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Multiplexed live-cell imaging for drug responses in patient-derived organoid models of cancer

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Setting up imaging parameters for a single focal plane of view analysis (Bright Field/Digital Phase Contrast Images): This section details how to generate a protocol that will allow for bright field kinetic imaging (converted to Digital Phase Contrast) in a single focal plane of view to determine PDO growth over time. The reason why users may choose to image in a single focal plane rather than generating a Z-Stack projection is because if the seeding density is too high, the PDOs overlap in different focal planes. This will then make it difficult for the analysis software to differentiate individual PDOs from each other.

1. Launch Gen5 software to begin imaging the 96-well plate.

2. **Click** New Task > Instrument Control > Incubate.
    1. Set the Requested temperature to 37 °C and **check** ON.
        1. *Note: Cytation will take a couple of minutes to reach temperature. Prior to placing the plate in the Cytation 5, make sure the reader is at 37 °C. This is necessary to maintain the sample at the appropriate temperature as well as decrease condensation on the lid, which will obstruct imaging.*
    2. **Close** the Instrument Control Panel.

3. Place plate in Cytation 5. **Click** New Task > Imager Manual Mode > Capture Now and input the following settings: Objective (select desired magnification); Filter (select microplate); Microplate format (select number of wells); and Vessel Type (select plate type). **Click** "Use Lid" and "Use slower carrier speed." **Click** OK.
    1. *Note for Vessel Type: Be as specific as possible when selecting information about the plate because the software is calibrated to the specific distance from the objective to the bottom of the plate for each plate type as well as the thickness of the plastic.*
    2. *Note for Slower Carrier Speed: Select this box to avoid disrupting organoid domes when loading/unloading plates.*

4. Identify the focal plane.
    1. **Select** a well of interest to view (left panel, below histogram).
    2. **Select** the Bright Field channel (left panel, top).
    3. Use the coarse and fine adjustment arrows (left panel, middle) to change the focal plane in view.
        1. *Note: The distance at which each tick changes the focal height, for both coarse and fine adjustment, can be lowered or increased using the sliders under the Focus drop down menu.*
    4. Identify the bottom and top focal heights of the domes and choose the focal height that falls in the middle of these two values.
        1. *Note: For users using Agilent 96-Well Plates and seeding 5 µL domes, this focal height will be approximately 3700 µm.*
    5. To ensure that the focal height settings are appropriate for other wells of interest, **select** another well (left panel, below histogram) and visualize this focal height to

make sure the image is still in focus. This is done by manually entering the focal positions. **Click** on the three dots next to the fine adjustment (left panel, top). A window will open. Type in the desired focal height.

5. Set the exposure settings for the Bright Field channel.
    1. First **Click** Auto Expose (left panel, top, under coarse and fine adjustment) to automatically determine an exposure that the Cytation 5 deems appropriate.
        1. If this exposure appears too dim or too bright, this can be adjusted manually using the plus and minus buttons on either side of the Auto Expose button.

6. Generate a template image from which the protocol/experiment will be based.
    1. **Click** on the "Camera" icon (left panel, bottom corner) to take a template image. This is what the images will look like when carrying out actual experiments.
    2. **Click** the "Process/Analyze" button to the right of the "Camera" icon.
    3. **Click** "Image Set" drop down menu (top left of the screen) and **click** on "Create experiment from this image set". A new Procedure window will open.
        1. *Note: The parameters selected for the image will automatically be taken into the new window whereby an experimental protocol can be created.*

7. Create Protocol.
    1. Set the temperature and gradient: **Click** on Set Temperature under the Actions heading (left). A new window will open. **Select** "Incubator On" and manually enter the desired temperature under "Temperature." Next, under "Gradient", manually enter "1." Close window by **selecting** OK.
        1. *Note: creating a 1 °C gradient will prevent condensation from forming on the lid of the plate.*
    2. Designating wells to image
        1. **Double click** on the Image tab under description.
        2. **Click** "Full Plate" (right corner, top). This will open the Plate Layout window.
        3. Highlight wells of interest using the cursor. **Click** OK.
        4. If desired, **check** "Autofocus binning" and "Capture binning" boxes. **Click** OK to close window.
            1. Note: Please refer to *Data Management* in the Discussion for specific scenarios in which this feature may be used.
    3. Set intervals for kinetic imaging.
        1. **Click** on Options under the Other heading (left).
        2. **Check** the "Discontinuous Kinetic Procedure" box.
        3. Under Estimated total time, enter the run time for the experiment (e.g., 5 days). Under Estimated interval, enter the interval at which to image the plate (e.g., every 6 hours).
        4. **Click** "Pause after each run" to allow time for the plate to be transferred to the BioSpa incubator. Close window by **selecting** OK.
    4. Set up Data Reduction to generate Digital Phase Contrast Images. Converting Bright field images into Digital Phase Contrast images allows users to more accurately

create masks around objects of interest even when PDOs are undergoing cell death and blebbing, which can interfere with generation of masks around live/viable PDOs.

1. In the toolbar, **click** Protocol > Data Reduction > Digital Phase Contrast, which will open a new tab.
2. Make sure the "Channel" is set to Bright Field and set the "Structuring Element Size" to the average size at which PDOs are expected to grow. **Click** "OK" to close the window, and then **click** "OK" again to close the Data Reduction window.

5. Save the Protocol.
    1. In the toolbar, **Click** File tab > Save Protocol as.
    2. **Select** location to save file. Enter file name. **Click** Save to close window.
    3. **Click** on the File > Exit in the toolbar. A tab will open to save changes to Imager Manual Mode. **Select** No.
    4. A tab will open to save changes to Experiment 1. **Select** No.
    5. A tab will open to update the protocol definition. **Select** Update.
    6. **Close** Gen5 software.

8. Import the Protocol into BioSpa OnDemand and finish setting up the Experiment.
    1. **Open** the BioSpa OnDemand software.
    2. **Select** an available slot in the BioSpa.
    3. Import the Protocol.
        1. Under the Procedure Info tab, **select** User in the drop-down menu.
        2. Next to Protocol slot, **click** Select > Add a new entry.
        3. Next to Protocol slot, **click** Select. This will open a new window to navigate to the desired Protocol in the file architecture.
        4. **Click** Open to import the Protocol into the BioSpa OnDemand software.
        5. Enter the amount of time needed to image the plate. **Click** OK to close the Gen5 Protocol List window.
            1. *Note: This step is especially important when running several experiments at a time. To determine the time needed to image the, click "Perform a timing run now." Click OK.*
    4. Set imaging intervals and schedule the experiment.
        1. Under Interval, enter the imaging interval which was designated previously in *Step 5.4* of *Setting up Imaging Parameters.*
        2. Under Start Time Options, **select** "When available."
            1. *Note: A specific start time can be designated instead of running the protocol at the next available time.*
        3. Under Duration, **select** "Fixed" or "Continuous."
            1. *Note: Selecting Fixed duration will set a specific endpoint for the experiment and requires the user to designate an experimental timeframe. Continuous duration will allow the experiment to run with no endpoint and can only be ended by a user stopping the experiment.*
        4. **Click** Schedule plate/vessel. This will open the Plate Validation Sequence.
        5. A tab will open with the proposed first read time. **Click** Yes to accept this schedule.

5. Remove the plate from the Cytation 5. **Click** Open drawer to access the appropriate slot. Place plate in BioSpa. **Click** Close drawer.
    1. *Note: This step can be performed at any point once the Protocol has been created. However, the plate must be in the Cytation 5 if one wishes to perform a timing run.*

Digital Phase Contrast Image analysis in Gen5 software: Below we describe methods to analyze data from the Digital Phase Contrast images generated from the Bright Field images. Representative images are provided in **Supplemental Figure S4.**

1. Opening image analysis module.
    1. **Open** experimental file in Gen5 software. **Select** Plate > View from the toolbar.
    2. **Change** Data drop-down menu to Dig.Ph.Con.
    3. **Double click** on a well of interest.
    4. **Select** Analyze > "I want to setup a new Image Analysis data reduction step" > OK.

2. Cellular Analysis.
    1. Primary Mask
        1. Under ANALYSIS SETTINGS, **select** Type: Cellular Analysis and Detection Channel: Dig.Ph.Con. (left panel, center).
        2. **Click** Options. The Primary Mask and Count page will open. In the Threshold box, **uncheck** "Auto" and adjust the slider as necessary to include or exclude objects of interest.
            1. *Note: When analyzing images in the Bright Field channel, ensure that Background is set to Light and for Digital Phase Contrast channel use Dark.*
            2. **Check** both boxes "Split touching objects" and "Fill holes in masks."
            3. **Open** "Advanced Detection Options."
            4. **Select** "Background Flattening" and "Auto."
                1. *Note: The Rolling Ball Diameter is a pre-processing technique where the image is sampled to distinguish background noise from actual signal. The diameter is how much of the image is sampled.*
            5. **Set** "Image Smoothing Strength" to between 1 and 10 cycles of 3x3 average filter depending on how much background material there is.
                1. *Note: Image smoothing is used to further decrease the impact of background noise on the generation of the mask, it reduces the variability of background signal to allow for more accurate border identification and better special measurements.*
            6. **Set** the "Primary Mask" to "Use Threshold Mask" from the drop-down menu, then **select** "OK".
            7. Under Object selection, designate a minimum and a maximum object size (µm). Adjust as necessary to exclude cellular debris/single cells.
                1. *Note: PDO size may vary significantly between different models and types. Use the measuring tool*  *in the Gen5 software to determine the minimum and maximum PDO size thresholds for each model.*
                2. **Deselect** "Include primary edge objects" and "Analyze entire image." To limit the analysis to a certain region of the well, **click** "Plug." This will open the Image Plug Window. Using the drop-down menu, **select** Plug

shape and adjust size and position parameters to fit over the region of interest.

1. *Note: It is important to maximize the number of PDOs within the plug while also excluding PDO-free areas to minimize background. Designate a plug size that will consistently capture the majority of the objects of interest across replicates. Generating a plug that also excludes the edges of the dome is important as it excludes any objects that may appear distorted due to the refraction of light from the extreme curvature of the dome around the edges.*

2. Subpopulation Analysis.
    1. **Click** on Calculated Metrics in the Cellular Analysis toolbar. **Click** "Select or create object level metrics of interest" (right corner, bottom). Under Available object metrics, **select** metrics of interest (e.g., Circularity, StdDev) and **click** the Insert button. **Click** OK.
        1. *Note: Morphology and density of each PDO model will determine the best metrics of interest to distinguish the subpopulation; for analysis of Digital Phase Contrast images, circularity and StdDev are the typical metrics of use. Circularity allows for exclusion of cellular debris that do not have a more typical uniform circular structure. StdDev is distinguishes between cellular debris and PDOs. Specifically, debris will appear uniformly bright whereas PDOs will have brighter edges and darker cores and therefore a high StdDev of light.*
    2. **Open** the Subpopulation Analysis page from the Cellular Analysis toolbar. **Click** Add to create a new subpopulation. A pop-up window will open.
    3. If desired, enter a name for the subpopulation. Under Object metrics, **click** on metric of interest and **press** Add Condition. In the Edit Condition window, enter parameters for the chosen Object metric. Repeat with additional metrics as necessary.
        1. *Note: Parameters may be adjusted manually (i.e., include all objects with a circularity greater than 0.3).*
    4. In the table at the bottom of the window, **check** the desired results to display. **Click** OK > Apply.
    5. To view the objects within the subpopulation, use the Object details drop-down menu to **select** the subpopulation. Objects that fall within the parameters will be highlighted in the image.
    6. To adjust subpopulation parameters, **reopen** the Subpopulation Analysis window from the Cellular Analysis toolbar. **Select** the subpopulation and **click** Edit.
    7. **Click** ADD STEP.
        1. *Note: This will apply the same analysis to all wells within the experiment at all time points. In the drop-down menu on the Matrix page, different metrics can be selected for individual viewing.*

End of treatment cell viability and cell death fluorescence imaging using Nexcelom Bioscience ViaStain™ AOPI Staining Solution: This section details the experimental procedure and parameters used to analyze cell viability and cell death within the organoid cultures using

fluorescence. AOPI is a combination of two reagents, acridine orange (AO) and propidium iodide (PI). AO can enter both live and dead cells, resulting in the staining of all nucleated cells; AO generates a green fluorescent signal. PI can only enter cells with compromised membranes, resulting in staining all dead nucleated cells; PI generates a red fluorescence signal. Due to Förster resonance energy transfer (FRET), the PI signal quenches the AO signal in cells stained with both dyes, resulting in no spill-over and no double positive results.

1. Addition of "Viastain™ AOPI Staining Solution" to PDO culture.
    1. Add AOPI staining solution at a 1:50 v/v ratio (e.g., 2 µL of staining solution to 100 µL culture medium) to each well being careful not to introduce any air bubbles.
    2. Gently shake the plate to mix the AOPI solution with the culture medium and incubate in a dark place for 25 minutes before continuing.
        1. *Note: Future experiments should be incubated with the AOPI solution for 30 minutes before reading.*

2. To set up a new protocol for the AOPI analysis, repeat steps 1-6 from "Setting up imaging parameters for a single focal plane of view analysis (Bright Field/Digital Phase Contrast Images)."

3. Create Protocol.
    1. Set the temperature and gradient: **Click** on Set Temperature under the Actions heading (left). A new window will open. **Select** "Incubator On" and manually enter the desired temperature under "Temperature." Next, under "Gradient", manually enter "1." Close window by **selecting** OK.
        1. *Note: creating a 1 °C gradient will prevent condensation from forming on the lid of the plate.*
    2. Formatting the plate layout and read description.
        1. **Double click** on the Image tab under Description.
        2. **Click** "Full Plate" (right corner, top). This will open the Plate Layout window.
        3. Highlight wells of interest that you wish to image using the cursor. **Click** OK.
        4. Under the image drop down menu (top, middle) **select** "Crop 75%."
            1. *Note: Selecting the "crop 75%" option reduces the amount of background fluorescence that will naturally occur around the edges of the wells as the field of view being imaged is slightly decreased.*
        5. **Select** both "Autofocus binning" and "Capture binning."
        6. Under "Channels" there should be one current channel selected "Bright Field;" **select** the number "2" to add a second channel.
            1. Under the Color drop down menu **select** "GFP 469,525."
            2. **De-select** "Auto" and then **click** on the microscope icon next to "Auto."
                1. *Note: This step will allow for manual setting of the exposure settings.*
            3. Adjust the "Illumination intensity," "Integration time" and "Camera gain" to appropriate values so that exposure levels are correct.

7. Repeat the previous step (3.2.6) but instead **select** the number "3" to add a third channel, and under the color drop down menu **select** "Texas Red 586,647."
8. Once the three channels are set up, **click** "OK" to close the "Imaging Step-Inverted Imager" tab.

3. **Click** "Validate" at the bottom of the "Procedure" window to confirm the procedure step sequence is valid and then **click** "OK" and then "OK" again.
    1. *Note: Raw images that are generated through this protocol will naturally have a lot of background fluorescence and therefore a Data Reduction>Image Preprocessing step needs to be implemented to normalize for background fluorescence.*

4. **Click** on the "Protocol" tab (top left) and **select** "Data Reduction."
    1. Under "Image processing" **select** "Image Preprocessing." A new window will open.
        1. *Note: The bright field image will not need any image preprocessing steps applied.*
    2. **De-select** "Apply image preprocessing" for the Bright Field channel.
    3. **Click** on the "GFP 469,525" tab.
        1. Make sure "Apply image preprocessing" is selected.
        2. **Select** "Dark" from the Background drop down menu.
        3. **De-select** "Use same options as channel 1."
        4. Make sure "Background Flattening" and "Auto" is selected.
        5. Change the "Image smoothing strength" to 1 Cycle of 3x3 average filter.
    4. **Click** on the "Texas Red 586,647" tab and repeat steps 4.3.1-5.
    5. **Click** "OK" and then **click** "OK" again.

5. Save the protocol for future use by **clicking** on the "File" tab, top left of the screen, and then "Save Protocol As…"
    1. Name the protocol appropriately and **click** "Save."

End of treatment cell viability and cell death fluorescence image analysis in Gen5 software: Below we describe methods to analyze data from the End of Treatment AOPI Fluorescence Protocol. Two separate image analysis steps need to be set up: 1) GFP channel, which is a measure of viability (AO); 2) Texas Red channel, which is the measure of cell death (PI).

1. Opening image analysis module.
    1. **Open** experimental file in Gen5 software. **Select** Plate > View from the toolbar.
    2. **Change** Data drop-down menu to Picture [Tsf[Bright Field+GFP 469,525+Texas Red 586,647]].
    3. **Double click** on a well of interest.
    4. **Select** Analyze > "I want to setup a new Image Analysis data reduction step" > OK.

2. Cellular Analysis for Cell Viability (Acridine Orange and GFP fluorescent channel).
    1. GFP Primary Mask
        1. Under ANALYSIS SETTINGS, **select** Type: Cellular Analysis and Detection Channel: Tsf[GFP 469,525] (left panel, center).

2. **Click** Options. The Primary Mask and Count page will open. In the Threshold box, **check** "Auto" and adjust the slider as necessary to include or exclude objects of interest.
    1. *Note: When analyzing images using the GFP or Texas Red channels, set the background to dark.*
2. **Select** "Split touching objects" and "Fill holes in masks."
3. **Open** "Advanced Detection Options."
    1. **Select** "Background Flattening" and **de-select** "Auto."
        1. *Note: The Rolling Ball Diameter is a pre-processing technique where the image is sampled to distinguish background noise from actual signal. The diameter should be set to roughly the size of the largest object being analyzed.*
    2. **Set** "Image Smoothing Strength" to 1 cycle of 3x3 average filter.
    3. **Set** the "Primary Mask" to "Use Threshold Mask" from the drop-down menu, and then **select** "OK."
    4. Under Object selection, designate a minimum and maximum object size (µm). Adjust as necessary to exclude cellular debris/single cells.
        1. *Note: PDO size may vary significantly between different models and types. Use the measuring tool*  *to determine the minimum and maximum PDO size thresholds for each model.*
        2. **Deselect** "Include primary edge objects" and "Analyze entire image." To limit the analysis to a certain region of the well, **click** "Plug." This will open the Image Plug Window. Using the drop-down menu, **select** Plug shape and adjust size and position parameters to fit over the region of interest.
        3. *Note: It is important to maximize the number of PDOs within the plug while also excluding PDO-free areas to minimize background. Designate a plug size that will consistently capture the majority of the objects of interest across replicates. Generating a plug that also excludes the edges of the dome is important as it excludes any objects that may appear distorted due to the refraction of light from the extreme curvature of the dome around the edges.*
4. Subpopulation Analysis.
    1. **Click** on Calculated Metrics in the Cellular Analysis toolbar. **Click** "Select or create object level metrics of interest" (right corner, bottom). Under Available object metrics, **select** metrics of interest (e.g., Circularity, Integral[Tsf[GFP 469,525]]) and **click** the Insert button. **Click** OK.
        1. *Note: Morphology and density of each PDO model will determine the best metrics of interest to distinguish the subpopulation. For analysis of the GFP channel, circularity is the only metric required as only viable material will fluoresce green and therefore there is no need for exclusion of debris.*
    2. **Open** the Subpopulation Analysis page from the Cellular Analysis toolbar. **Click** Add to create a new subpopulation. A pop-up window will open.
    3. If desired, enter a name for the subpopulation. Under Object metrics, **click** on metric of interest and **select** Add Condition. In the Edit Condition window, enter parameters for the chosen Object metric. Repeat with additional metrics as necessary.
        1. *Note: Parameters may be adjusted manually (i.e., include all objects with a circularity greater than 0.3).*

4. In the table at the bottom of the window, **select** the desired results to display. **Click** OK > Apply.
5. To view the objects within the subpopulation, use the Object details drop-down menu to **select** the subpopulation. Objects that fall within the parameters will be highlighted in the image.
6. To adjust subpopulation parameters, **reopen** the Subpopulation Analysis window from the Cellular Analysis toolbar. **Select** the subpopulation and **click** Edit.
7. **Click** ADD STEP.
    1. *Note: This will apply the same analysis to all wells within the experiment at all time points. In the drop-down menu on the Matrix page, different metrics can be selected for individual viewing.*

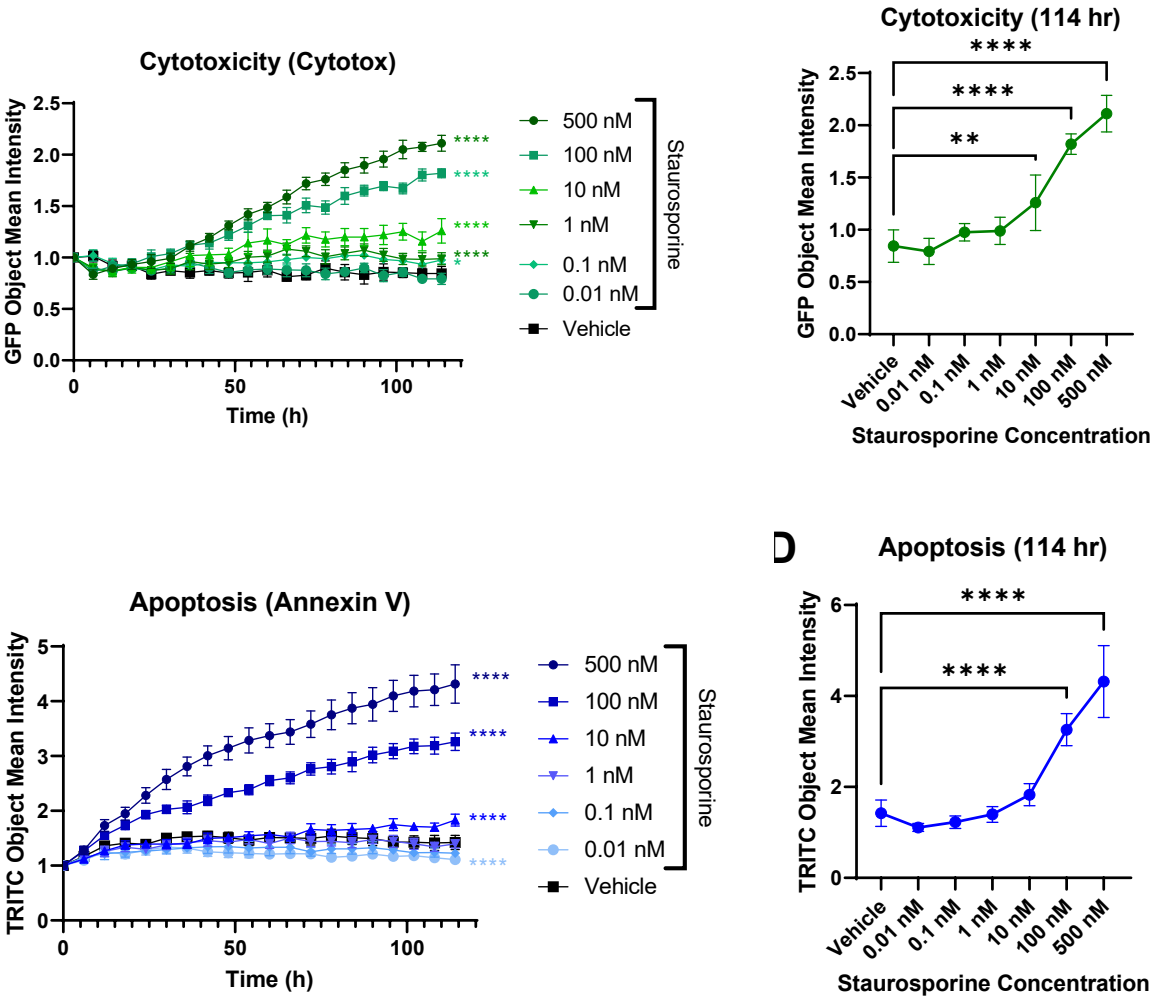
3. Cellular Analysis for Cell Death (Propidium Iodide and Texas Red fluorescent channel).
    1. Texas Red Primary Mask.
        1. Under ANALYSIS SETTINGS, **select** Type: Cellular Analysis and Detection Channel: Tsf[Texas Red 586,647] (left panel, center).
        2. **Click** Options. The Primary Mask and Count page will open. In the Threshold box, **uncheck** "Auto" and set the value to 5000.
            1. *Note: When analyzing images using the GFP or Texas Red channels, set the background to dark.*
    2. **Check** both boxes "Split touching objects" and "Fill holes in masks."
    3. **Open** "Advanced Detection Options."
        1. **Select** "Background Flattening" and **select** "Auto."
        2. **Set** "Image Smoothing Strength" to 0 cycles of 3x3 average filter.
        3. **Set** the "Evaluate background on" to 85% of lowest pixels.
            1. *Note: This step is needed to ensure that no background specks of fluorescence are include in the mask for analysis.*
        4. **Set** the "Primary Mask" to "Use Threshold Mask" from the drop-down menu, then **select** "OK."
            1. *Note: For the Texas Red channel, minimal image preprocessing techniques are needed for the analysis since the fluorescence is more focal.*
    4. Under Object selection, designate a minimum and a maximum object size (µm).
        1. *Note: For the Texas Red channel analysis, the minimal object size should be approximately the size of one cell (e.g., 10 µm), but the maximum should still be that of the largest object expected for the given model.*
    5. **Deselect** "Include primary edge objects" and "Analyze entire image." To limit the analysis to a certain region of the well, **click** "Plug." This will open the Image Plug Window. Using the drop-down menu, **select** Plug shape and adjust size and position parameters to fit over the region of interest. This should be the same size and position as the plug used for the GFP channel.
        1. *Note: It is important to maximize the number of PDOs within the plug while also excluding PDO-free areas to minimize background. Designate a plug size that will consistently capture the majority of the objects of interest across replicates. Generating a plug that also excludes the edges of the dome is important as it*

excludes any objects that may appear distorted due to the refraction of light from the extreme curvature of the dome around the edges.

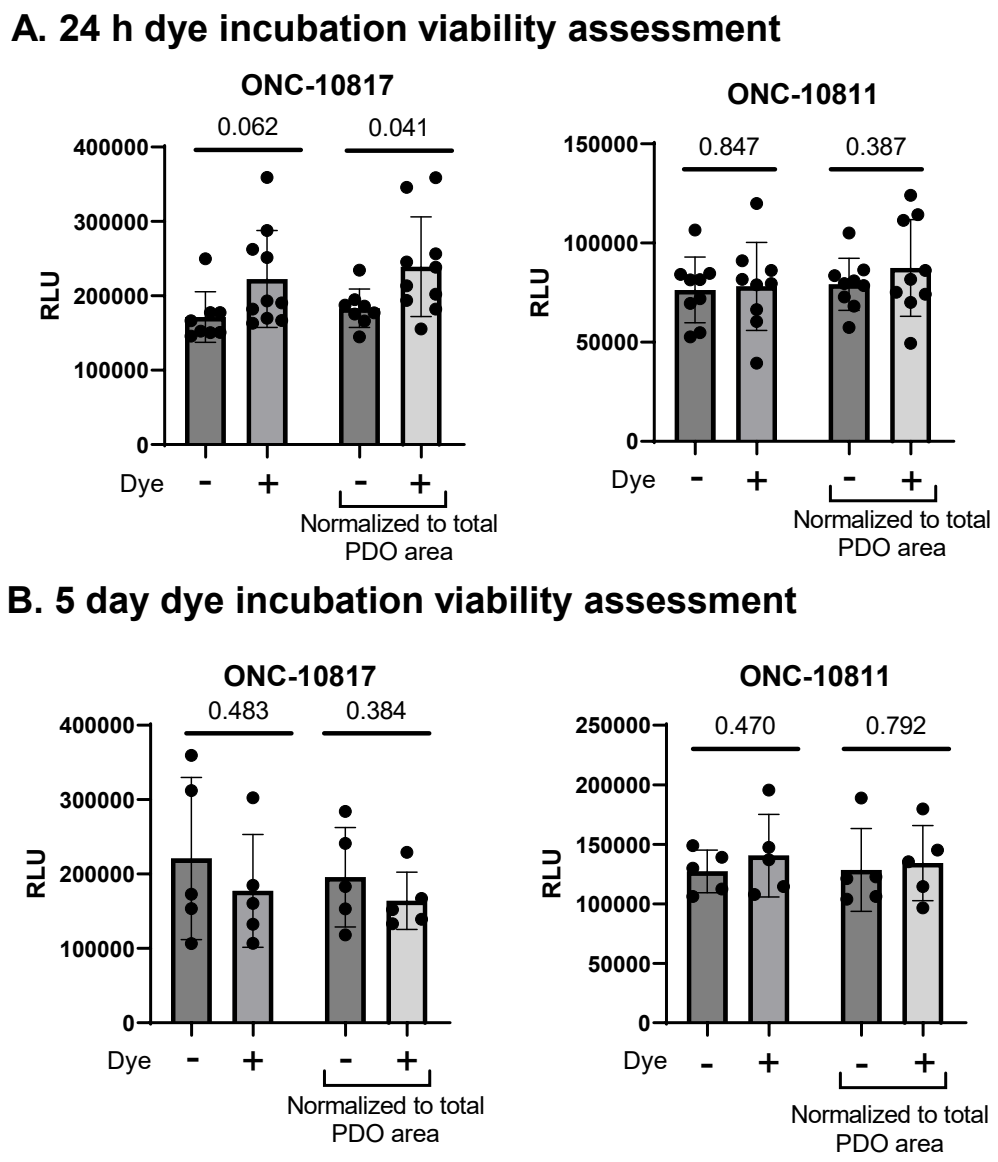
6. **Click** ADD STEP.
    1. *Note: This will apply the same analysis to all wells within the experiment at all time points. In the drop-down menu on the Matrix page, different metrics can be selected for individual viewing.*
    2. *Note: There is no need for a subpopulation analysis for the Texas Red channel as the majority of the signal will be focal and will stem from dead material. Therefore, this signal should not be excluded from analysis.*

Supplemental Table S1: Organoid Culture Media Components. Note that reagents have been optimized for culturing gynecologic cancer PDOs.

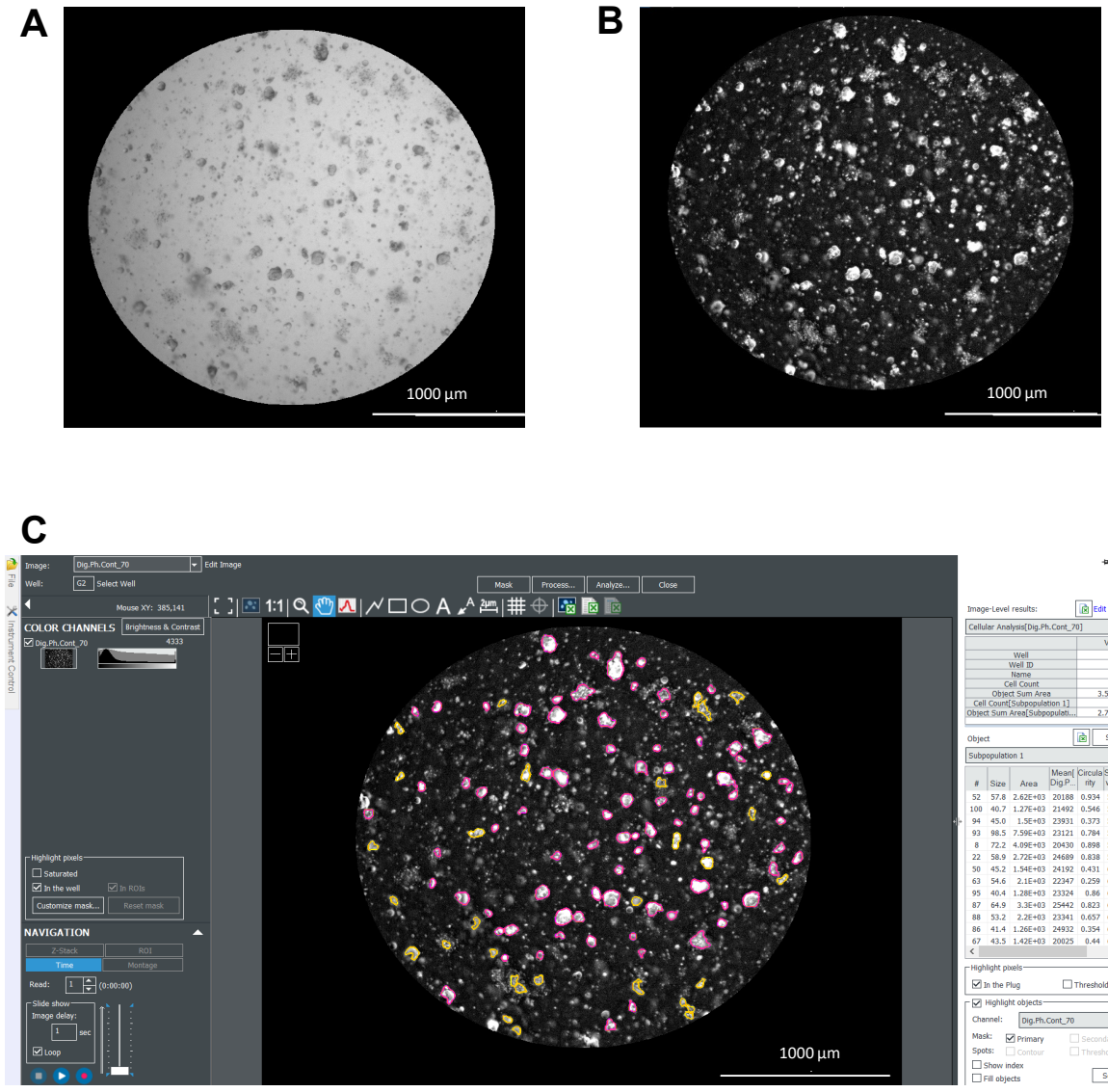
Organoid Culture Media Component	Final Concentration
AdDF+++ (Advanced DMEM/F12, Glutamax, HEPES, penicillin/streptomycin)	1X Glutamax, 10 mM HEPES, 10 units/mL penicillin/streptomycin
Noggin	100 ng/mL
Respondin-1	250 ng/mL
A83-01	500 nM
FGF-10	10 ng/mL
Heregulinβ-1	37.5 ng/mL
EGF	0.5 ng/mL
Estradiol	100 nM
Y-27632	5 µM
Forskolin	10 µM
Hydrocortisone	500 ng/mL
B27 supplement	1X
Nicotinamide	10 mM
N-acetylcysteine	1.25 mM
Primocin	100 µg/mL



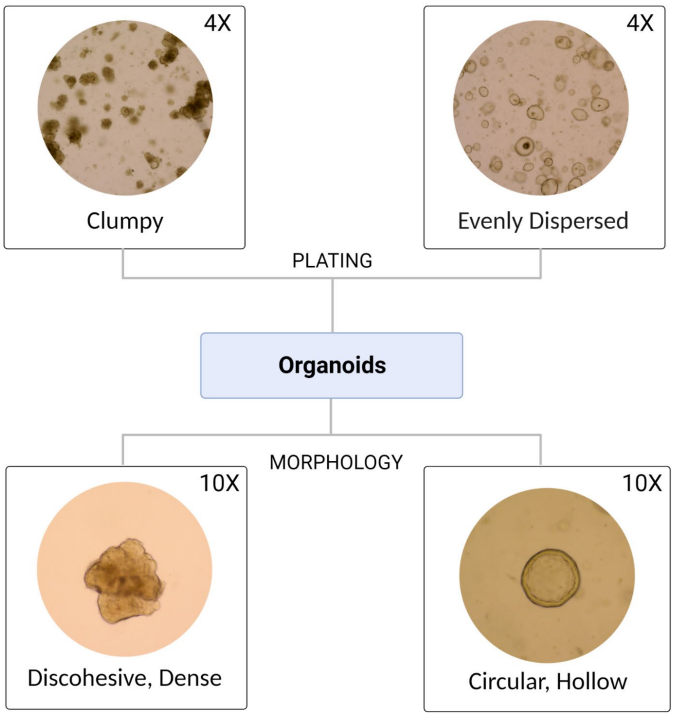
Supplemental Figure S1: Multiplexed live-cell imaging of ONC-10811. PDOs were plated in 96-well plates and incubated in Annexin V Red (1:400) and Cytotox Green (200 nM) dyes overnight at 37 °C. The following day, PDOs were treated with increasing concentrations of staurosporine and were imaged every 6 hours for 5 days. (A) Time and dose-dependent increase in cytotoxicity in response to staurosporine. Data were plotted as the Object Mean Intensity in the GFP channel. (B) Dose-response of staurosporine at 114 hrs. Data were plotted as the Object Mean Intensity values in the GFP channel at the 114 hr timepoint. (C) Time and dose-dependent increase in apoptosis in response to staurosporine. Data were plotted as the Object Mean Intensity in the TRITC channel. (D) Dose-response of staurosporine at 114 hrs. Data were plotted as the Object Mean Intensity values in the TRITC channel at the 114 hr timepoint. Data in A and C were normalized to PDO number at time 0 at the well level. N=5 technical replicates per treatment in each model. **** $p < 0.0001$ vs. vehicle control by 2-way ANOVA.



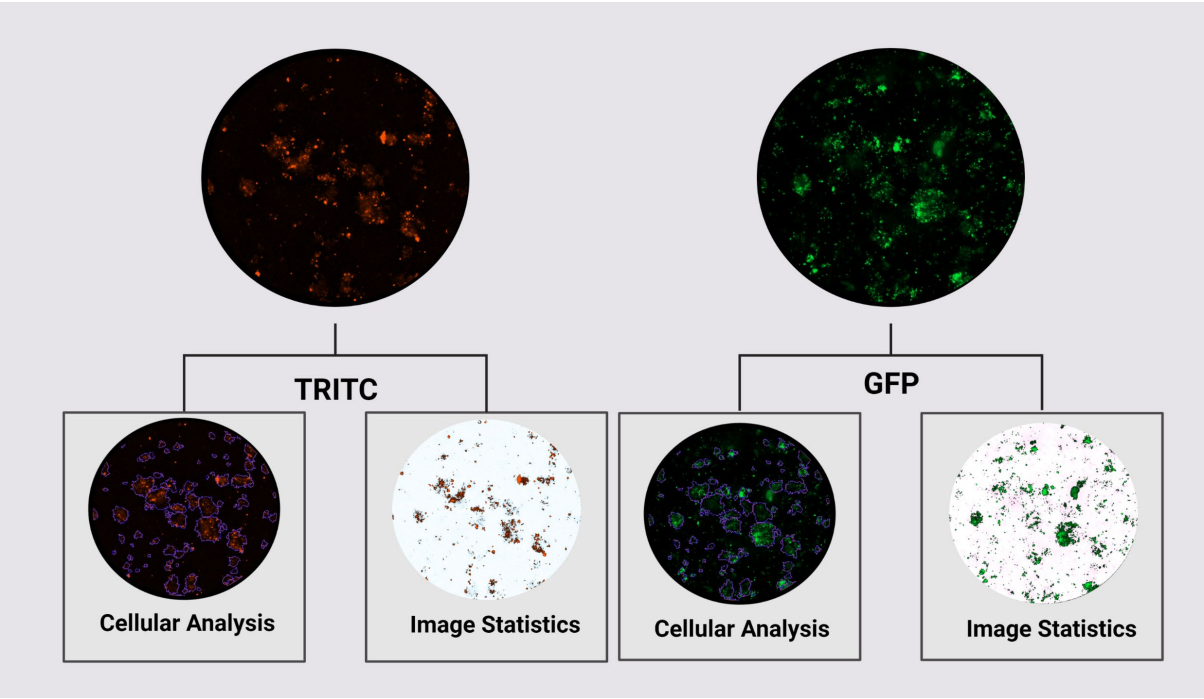
Supplemental Figure S2: Treatment with Annexin V and Cytotox does not perturb PDO viability. PDOs were plated in 96-well plates and incubated with Annexin V Red (1:400) and Cytotox Green (200 nM) dyes overnight at 37 °C. Following the 24 hour incubation, viability was assessed using the CellTiter-Glo 3D assay per the manufacturer's protocol. (**A**) Viability in dye-treated and untreated PDOs at the 24 hr timepoint. Both relative light unit (RLU) values and values normalized to organoid sum area are presented. Specifically, the CellTiter-Glo3D RLU for each well was normalized to the summation of organoid area for that well at the time of plating (i.e., immediately after dye addition). Total PDO area was determined using the "Sum Area" calculation in Cellular Analysis. N=10.(**B**) Viability in dye-treated and untreated PDOs at the 114 hr timepoint. Both raw luminescence values and values normalized to total PDO area are presented. The CellTiter-Glo3D RLU for each well was normalized to the summation of organoid area at 24 hours post-plating, which corresponds to time 0 in the kinetic imaging experiments. N=5. Significance in A and B was assessed using an unpaired t-test; p values are listed on the graphs.



Supplemental Figure S3: Label-free analysis of PDOs using digital phase contrast. This figure contains representative images (2.5X objective) depicting label-free analysis as described in the **Supplemental Protocols:** *Setting up imaging parameters for a single focal plane of view analysis (Bright Field/Digital Phase Contrast Images) and Digital Phase Contrast Image analysis in Gen5 software*. (**A**) Example Bright Field image of a prostate cancer PDXO model at a single focal plane. (**B**) The Bright Field image in **A** was converted to a Digital Phase Contrast image. Dark objects in the Bright Field image appear bright in the Digital Phase Contrast image and vice versa. (**C**) Example of PDO masking using the Digital Phase contrast image. Note that the edges of objects of interest become much more defined as compared to the Bright Field images. In this representative image, objects in the primary mask are outlined in yellow. The subpopulation in (C) (outlined in pink) is defined based on circularity (>0.3), area (>1000), Mean[Dig.Ph.Con] > 2000, StdDev[Dig.Ph.con] > 5000 + < 13500, and Peak[Dig.Ph.Con] > 12500. The parameters used in this representative figure were applied to data in Figure 8 to normalize to cell count on day 0.



Supplemental Figure S4: PDO models may vary in their morphology and plating consistency. *Upper Panel*: Examples of differential dispersion of PDOs in the BME domes. *Lower Panel:* Representative images of discohesive vs. circular PDOs. All images were acquired with an EVOS microscope. Magnifications are noted.



Supplemental Figure S5: Example images using Cellular Analysis vs. Image Statistics for quantifying fluorescence. Using Cellular Analysis, users can define specific populations within an image and measure fluorescence in those regions. Image Statistics may also be used to measure fluorescence in an image by defining a threshold to exclude background signal. *See Discussion for the limitations of using Image Statistics.* Images are at 4X magnification.