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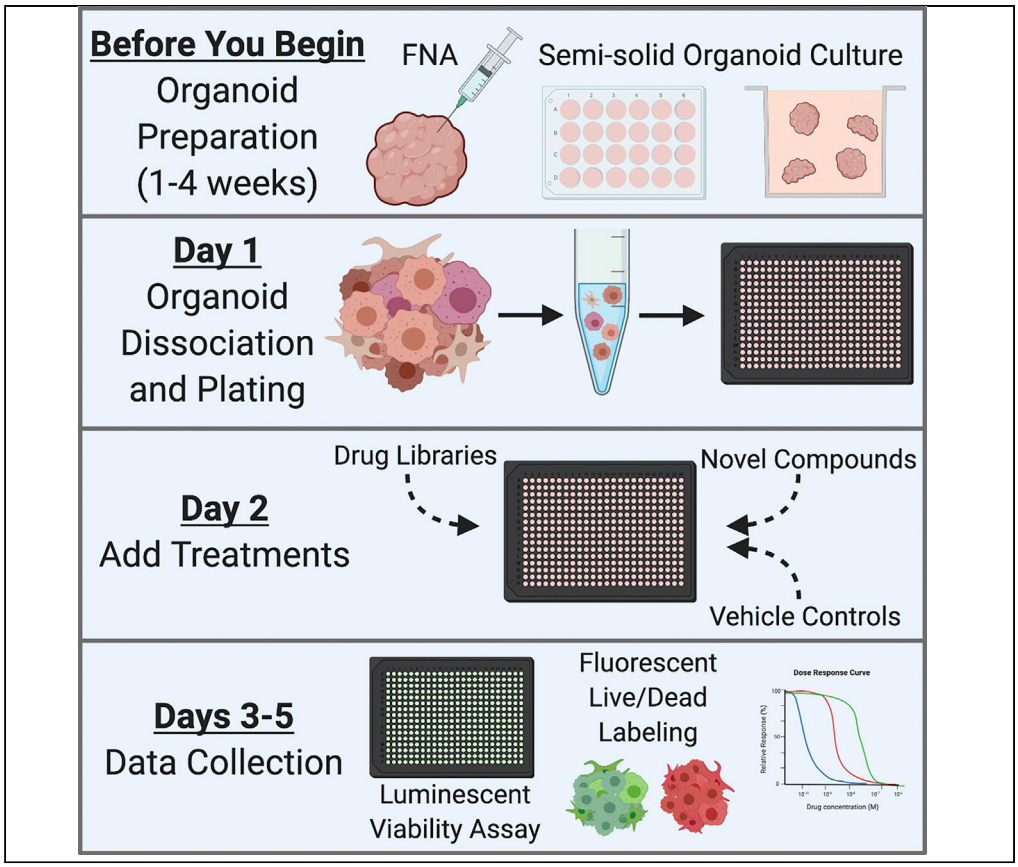
High-throughput drug screening of fine-needle aspiration-derived cancer organoids



Generation of fine-needle aspiration (FNA)-derived cancer organoids has allowed us to develop a number of downstream applications. In this protocol, we start with organoids cultured in a semi-solid format. We dissociate organoids into single cells and then plate in a 384-well format for high-throughput drug screening. While this method must be fine-tuned for each individual organoid culture, it offers a format well suited for rapidly screening medium-sized drug/compound libraries (500–5,000 molecules) and generating dose-response curves to measure relative efficacy.

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HIGHLIGHTS

Protocol to dissociate organoids into single cells for high-throughput plating methods

Organoids form in a 384-well format when plated in cell-repellent plates

Steps for high-throughput drug screening with viability assays

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High-throughput drug screening of fine-needle aspiration-derived cancer organoids

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SUMMARY

Generation of fine-needle aspiration (FNA)-derived cancer organoids has allowed us to develop a number of downstream applications. In this protocol, we start with organoids cultured in a semi-solid format. We dissociate organoids into single cells and then plate in a 384-well format for high-throughput drug screening. While this method must be fine-tuned for each individual organoid culture, it offers a format well suited for rapidly screening medium-sized drug/compound libraries (500–5,000 molecules) and generating dose-response curves to measure relative efficacy.

For complete details on the use and execution of this protocol, please refer to Lee et al. (2020) and Vilgelm et al. (2020).

BEFORE YOU BEGIN

Organoid preparation

Timing: 1–4 weeks

1. Fine-needle aspiration should be performed on the tumor tissue of interest per standard protocols and grown in a semi-solid format consisting of complete organoid media plus 5% Matrigel as described in Vilgelm et al. (2020) and Phifer et al., 2020.

Note: The number of cells needed is dependent on the growth rate of the organoids over 5 days. Slow-growing organoids will require more cells to be plated initially to preserve the time course of the experiment. We have found that for most cell lines, 12 wells of semi-solid organoid culture (3–10 organoids/well depending on organoid size) are sufficient to plate one 384-well plate.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Hoechst	Invitrogen	H3570
Propidium iodide	Invitrogen	P3566
Calcein AM	Invitrogen	65-0853
Dulbecco's modified Eagle's medium (DMEM)	Sigma	D6429-500ML
Nutrient Mixture F-12 Ham (Ham's F12)	Sigma	N4888-500ML
MCDB 105 medium, unmodified	Cell Applications	117-500
Penicillin-streptomycin	Sigma	P4333-100ML
L(+)-Glutamine 200 mM 100×	VWR	VWRL0131-0100
Normocin	InvivoGen	ant-nr-1
Fetal bovine serum (FBS)	Thermo Fisher Scientific	26140079
B-27 supplement	Gibco	17504044
Matrigel	Fisher Scientific	CB-40234
TrypLE Express Enzyme, 1×	Gibco	12604013
Cell culture phosphate buffered saline (PBS), 1×, without calcium and magnesium	Corning	21040CV
Critical commercial assays		
CellTiter-Glo 3D cell viability assay	Promega	G9681
Software and algorithms		
Excel	Microsoft Office	n/a
Prism 8	GraphPad	n/a
Other		
384-well cell-repellent plate, PS, sterile, flat bottom, black, µClear, w/lid	Greiner-BioOne	781976
Reagent reservoir	n/a	n/a
ImageXpress Micro XL automated high-content microscope	Molecular Devices	n/a
Bravo automated pipette liquid transfer system	Velocity 11/Agilent	n/a
Synergy NEO multi-mode plate reader	BioTek	n/a
EL406 plate washer and bulk dispenser with wide-bore tip 10 µL cassette	BioTek	n/a

MATERIALS AND EQUIPMENT

Alternatives: While we recommend using the automated cell plating method described, a multi-channel pipette can also be used to plate cells and add drug. However, manual plating may not be as reliable and accurate as automated methods, contributing to inconsistent organoid growth and high variation in measurements.

Complete DMEM

Reagent	Final concentration (%)	Amount
DMEM	44%	250 mL
Ham's F12	22%	125 mL

(*Continued on next page*)

Continued

Reagent	Final concentration (%)	Amount
MCDB	22%	125 mL
FBS	10%	56 mL
L-Glutamine	0.9%	5.6 mL
Penicillin-streptomycin	0.9%	5.6 mL
Normocin	0.2%	1.2 mL
Total	100%	568.4 mL

STEP-BY-STEP METHOD DETAILS

Organoid dissociation

Timing: 1 h

While it would be optimal to plate intact organoids, issues with size inconsistency and fragility currently make this unrealistic to do in high-throughput. With dissociation into single cells, patient-derived cancer organoids can be easily plated and allowed to reform in each well of a 384-well plate.

1. Collect and wash organoids
   a. With a P1000 tip, pipette semi-solid cultures repetitively in a circular motion to break up the Matrigel.
   b. Transfer cultures to a 15 mL conical tube.
   c. Centrifuge at 340 × *g* for 5 min at 20°C–25°C and aspirate media.

   Note: This will be the standard centrifugation step for the remainder of the organoid dissociation.
   d. Wash pelleted organoids with 10 mL cold PBS to remove any residual Matrigel or media, centrifuge as in step 1c, aspirate as much PBS as possible without disturbing the pellet.

2. Dissociate organoids in TrypLE
   a. Resuspend organoids in 5 mL TrypLE and incubate at 37°C for 30 min, with vigorous pipetting every 10 min to break up cell clumps.

   Note: If the organoids are large, you may need to pipette with a P1000 tip every 5 min to enhance dissociation. Troubleshooting 1.
   b. When close to single cell suspension or small clumps, centrifuge as in step 1c.
   c. Aspirate TrypLE, then wash in warm modified DMEM media and centrifuge again as in step 1c.

Plating organoids in a 384-well format

Timing: 1 h

Once dissociated, these cells can be plated with established cell suspension plating methods. We utilize two formats of plating, depending on the number of plates needed for the given experiment. The first, which involves plating by hand, is for a small number of plates ($\leq$ 3) or for those who do not have access to high-throughput plating equipment. The other is automated and can be used for large-scale, high-throughput experiments.

3. Count cells and prepare media
   a. Count cells and resuspend to a concentration of 9,500 cells/mL in complete DMEM + 2% Matrigel in a 50 mL conical tube.

Note: We have successfully used 5% Matrigel as well but find that 2% is sufficient for our cultures and purpose. Using higher percentages of Matrigel, in combination with the optional centrifugation step following plating, may allow for enhanced downstream imaging.

⚠ CRITICAL: Although this is a low concentration of Matrigel, the cell suspension will still solidify at ambient temperature within minutes, if not kept on ice. The Matrigel should not be added until right before plating, and the cell suspension should be kept on ice prior to and while plating the cell suspension. Troubleshooting 2.

Note: We have found that 285 cells per well is an ideal starting concentration for the majority of our organoids. However, for particularly slow-growing cultures, we have increased the number up to 1,000 cells per well. We have not had to decrease the number of initial cells below 285/well for any of our cultures to date. Troubleshooting 3.

4. Plate cells
   a. Equip BioTek EL406 peristaltic pump with a 10 μL wide-bore tip cassette and set to low speed

Note: Dead volume is the excess volume required by a liquid handling instrument. It can be an unusable portion (e.g., residual volume in labware that is not accessible to the instrument) or a used portion that is not transferred to the assay container, such as the volume needed to prime tubing/tips. To decrease dead volume, reduce pre-dispense volume as much as possible. To decrease cross-well dispensing, minimize the height of the tips to the carrier to as low as 1 mm. Dispense from tips should appear as a drop rather than a stream.

   b. Swirl the tube of cells prior to priming the tubing and before each plate to ensure equal suspension dispensing. Be careful not to introduce air bubbles.
   c. Dispense 30 μL cell suspension per well into the cell-repellent microplates.

Note: For smaller-scale experiments, cells can be plated using a manual or electronic (repeating) multi-channel P200 pipette. Transfer the cell suspension + Matrigel to an appropriately sized reservoir for the volume size. If possible, place the reservoir on ice to prevent solidification. Avoid introducing bubbles into the media when pipetting.

Optional: Following cell plating, centrifuge the plate(s) at 220 × *g* for 1 min to allow cells to be pushed toward the bottom of the well. This helps to bring the cells and clusters (along with the Matrigel) into similar z-planes, which helps to stabilize the cultures for both downstream applications of microscopy and/or refreshing media or adding reagents to the wells.

   d. Allow organoids to form for at least 24 h in a 37°C, 5% CO_2 incubator

Drug screening

Timing: 1–4 days

Once the organoids are plated, they can be used for a number of different screens. We have used this format for primary screening of a large drug library and collection of dose-effect data for multiple drugs in a single experiment. Likewise, this could be modified for functional genomics screens (e.g., CRISPR, siRNA, etc.), phenotypic screening (e.g., high-content imaging), or other cellular read outs or functional assays.

5. Drug screening

a. Dispense 10 μL of treatment at 4× the desired concentration (bringing total well volume to 40 μL) to wells of each plate, including replicates and appropriate vehicle controls.

Note: If using DMSO, maximum DMSO concentration should be determined empirically for any organoid lines prior to drug screening. For all organoids we have screened to date, our DMSO concentrations do not exceed 0.1%.

i. For large screens, drug libraries may be "stamped" into organoid plates utilizing a Bravo automated pipette liquid transfer system, which holds a 384-well tip head to simultaneously dispense reagents from a drug source plate to each organoid plate within seconds.

Note: Drugs may also be added using a multi-channel pipette if necessary.

b. Incubate plates at 37°C, 5% CO_2 for the desired treatment time (24–96 h)

6. Data collection and analysis

Note: Either method below can be used, however we do not recommend using both methods on the same cultures.

a. Fluorescent Imaging of Live/Dead Cells
   i. 24 h prior to the end of drug treatment, add propidium iodide to a final concentration of 5 μg/mL to the wells.
   ii. Incubate at 37°C for 24 h.
   iii. Add Hoechst to the wells at a final concentration of 10 μg/mL to the wells.
   iv. Incubate at 37°C for 2 h.
   v. Add Calcein AM to the wells at a final concentration of 5 μM.
   vi. Incubate for 1 h.
   vii. Image with a fluorescence microscope.

b. CellTiter-Glo 3D

Optional: Image organoids before adding CellTiter-Glo 3D

i. Using the Bravo liquid handler or a multi-channel pipette, add 40 μL CellTiter-Glo 3D to the wells and mix vigorously to break up and lyse organoids

Note: Check the wells under a microscope following this step to ensure that all organoids have been broken up. Repeat vigorous pipetting as necessary until none remain. Troubleshooting 4.

⚠ CRITICAL: Make sure not to introduce bubbles when mixing, as it will impact the readout from the plate reader and introduce errors in the data.

ii. Place plates on a plate shaker for 25 min (up to 800 RPMs).
iii. Centrifuge the plates to eliminate any bubbles and to draw all liquid to the bottom of the well.
iv. Quantify luminescence with a plate reader

Determine drug effect by subtracting the control luminescence from the treatment luminescence, then plot against drug concentration for each treatment using Excel or GraphPad Prism.

EXPECTED OUTCOMES

Please see Figures 1, 2, and 3 for the expected outcomes of this protocol.

LIMITATIONS

While this protocol has worked very well for the thyroid and melanoma organoid lines we have used, it may not work for every organoid line. Very large organoids are difficult to dissociate, due to low

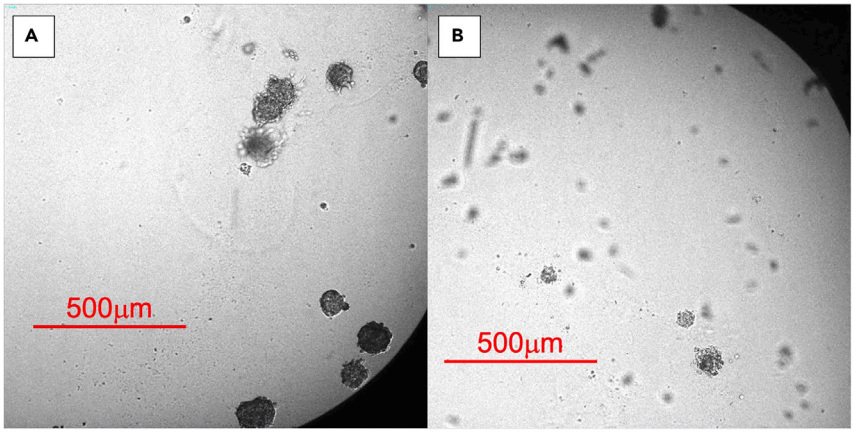

Figure 1. Bright-field images of thyroid organoids in a 384-well plate
(A) Healthy, untreated organoids formed over 4 days.
(B) Organoids treated with a high concentration of a cytotoxic drug for 72 h.

penetration of the TrypLE in 30 min. While the TrypLE can be left on longer, it may also cause cell death after prolonged exposure. This can also be an issue for organoids which are not facile; the TrypLE incubation may be too damaging for the cells to reform organoids and grow once plated. While we have not had any survival issues with our lines, a small sample of the culture should be tested with this protocol prior to plating an experiment to ensure that the cells can recover from dissociation.

As discussed in the protocol, the cell numbers listed herein are not concrete for every line. Some slow-growing lines need more cells initially to grow within the allotted experiment time. If you plan on doing a large screen with a line, we would recommend plating one trial plate to observe the growth over the treatment period (3–5 days) with multiple initial cell numbers so that the experiment can be optimized for the individual line you are screening.

TROUBLESHOOTING

Problem 1

Organoids are clumping/will not dissociate.

Potential solution

If starting with large organoids, frequent agitation may be needed to break down the cell clumps.

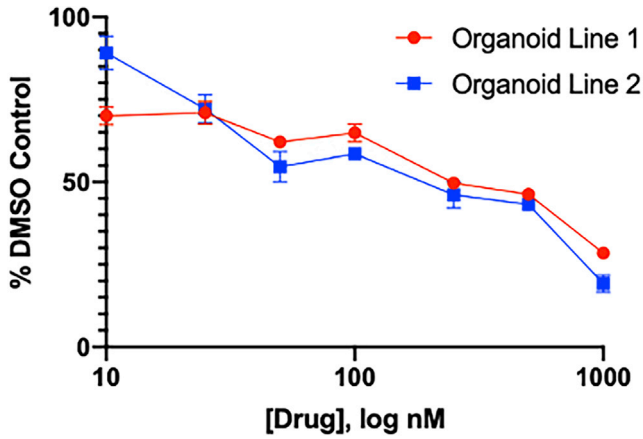

Figure 2. Sample dose-response curve generated from CellTiter-Glo 3D luminescence readout
Two different thyroid organoid cultures were treated with the same cytotoxic drug for 72 h.

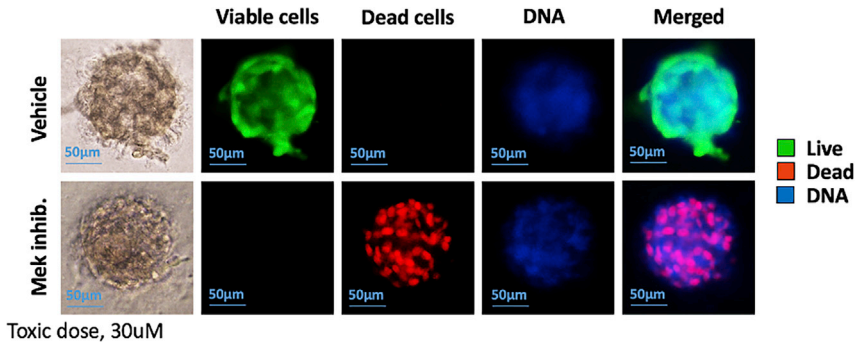

Figure 3. Fluorescent imaging to identify live and dead cells

Patient-derived melanoma organoids were labeled with Calcein AM (live cells exhibit green fluorescence, dead cells are negative) and propidium iodide (dead cells exhibit red fluorescence, live cells are negative). To visualize both live and dead cells, a cell-permeable DNA stain (Hoechst 33342) was used. While overall organoid structure remained intact, the cells in the melanoma organoids were entirely killed following 2 days of treatment with a high dose (30 μM) MEK inhibitor.

If the organoids are all clumping together, they can be placed on a shaker or rotator in the incubator to keep them in motion and discourage clumping.

If a significant amount of Matrigel or media is left before the TrypLE is added, this may also hinder dissociation. Make sure to aspirate as much as possible without disturbing the pellet and perform an extra PBS wash if needed.

Problem 2

Cell suspension is too viscous/Matrigel is solidifying.

Potential solution

Do not add the Matrigel until immediately before plating, and make sure to keep the media/cell suspension on ice once the Matrigel is added if possible. Once the Matrigel completely solidifies, it cannot be reversed in a manner that makes it reusable.

Problem 3

Organoids are small/not many form.

Potential solution

If the organoids are small or infrequent, more cells may need to be plated in each well initially. If the cell number has already been scaled up and the organoids are still small or not forming, it may be reflective of cell death due to dissociation or the plating method used. Try reducing the dissociation time and switching to a gentler plating method, such as using a multi-channel pipette. It is also important to note that organoid growth patterns are relatively consistent from 24- to 384-well plates. If an organoid line forms small organoids in the 24-well semi-solid format, it will also form small organoids in high-throughput settings.

Problem 4

Organoids remain after adding CellTiter-Glo 3D and mixing.

Potential solution

Adjust the settings on the Bravo or make sure that the pipette tips are going to the bottom of the well, and that it is being mixed as fast as possible. This step can be repeated, but take care not to introduce bubbles.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Vivian Weiss (vivian.l.weiss@vumc.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any datasets or code.

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AUTHOR CONTRIBUTIONS

K.B., C.P., and V.B. developed the protocol with guidance from D.W., A.V., E.L., and J.B. The manuscript was prepared by K.B. and reviewed by all authors. Funding for this project was provided by A.V., E.L., and V.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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