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Profiling sensitivity to targeted therapies in EGFR-mutant NSCLC patient-derived organoids

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TITLE:

Profiling Sensitivity to Targeted Therapies in EGFR-Mutant NSCLC Patient-Derived Organoids

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KEYWORDS:

Organoid, lung organoid, PDO, NSCLC, lung cancer, osimertinib, EGFR, targeted Inhibitor, resistance

SUMMARY:

This protocol describes a standardized evaluation of drug sensitivities to targeted signaling inhibitors in NSCLC patient-derived organoid models.

ABSTRACT:

Novel 3D cancer organoid cultures derived from clinical patient specimens represent an important model system to evaluate intratumor heterogeneity and treatment response to targeted inhibitors in cancer. Pioneering work in gastrointestinal and pancreatic cancers has highlighted the promise of patient-derived organoids (PDOs) as a patient-proximate culture system, with an increasing number of models emerging. Similarly, work in other cancer types has focused on establishing organoid models and optimizing culture protocols. Notably, 3D cancer organoid models maintain the genetic complexity of original tumor specimens and thus translate tumor-derived sequencing data into treatment with genetically informed targeted therapies in an experimental setting. Further, PDOs might foster the evaluation of rational combination treatments to overcome resistance-associated adaptation of tumors in the future. The latter focuses on intense research efforts in non-small-cell lung cancer (NSCLC), as resistance development ultimately limits the treatment success of targeted inhibitors. An early assessment of therapeutically targetable mechanisms using NSCLC PDOs could help inform rational

combination treatments. This manuscript describes a standardized protocol for the cell culture plate-based assessment of drug sensitivities to targeted inhibitors in NSCLC-derived 3D PDOs, with potential adaptability to combinational treatments and other treatment modalities.

INTRODUCTION:

Personalized therapies against oncogenic drivers have revolutionized cancer treatment, improving patient survival and reducing treatment-mediated side effects[1]. Recent advances in molecular diagnostics and sequencing technologies have highlighted the complexity of human tumors, with spatial and temporal heterogeneity impacting treatment response[2]. Recapitulating these subclonal differences in cell culture models has long been limited to investigating selected alterations of interest in otherwise uniform cell lines. Newly developed 3D PDO models generated from tumor biopsies or surgical tumor resections allow for improved representation of cellular complexity and signaling crosstalk within patient-derived tumor tissue[3]. As such, tumor organoids derived from gastrointestinal and pancreatic cancer have successfully been generated and recapitulate the genetic diversity and determinants of treatment response[4-6]. In non-small cell lung cancer (NSCLC), organoid development and establishment challenges are acknowledged, and optimization of culture techniques and selective media factors is needed to enable broader and more systematic use of NSCLC PDOs in the future[7,8].

Developing combinatorial therapies targeting residual tumor cells that withstand initial drug treatment is essential to inhibit resistance development and ultimately to improve patient survival[9]. Given the architectural complexity of organoid cultures, classical drug response parameters need to be optimized to allow for accurate and reproducible testing of drug sensitivities. Imaging-based readouts[10,11] and classical cell viability assays measuring cellular ATP abundance[6,12], amongst other techniques, are available to profile drug responses in PDO cultures. Here, we develop and describe a standardized protocol to evaluate drug sensitivities to targeted therapy against known clinical drivers in NSCLC PDO models.

PROTOCOL:

For research, all human tissue collection was approved by the UCSF Internal Review Board (IRB, protocol no.: #13-12492, CC#13-6512, and CC#17-658). The establishment of organoid cultures from de-identified clinical specimens was performed in collaboration with research partners according to previously published methods[13-16]. Organoid cultures were retrieved for maintenance and drug escalation experiments at passage three or later. All the following protocols were performed under aseptic conditions in a mammalian tissue culture laboratory environment.

[Place **Figure 1** here]

1. Experimental preparations

1.1. Prepare growth medium (GM) as previously reported[15]: Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture F12 (DMEM/F-12) with L-alanyl-L-glutamine, supplemented with 100 U/mL penicillin/streptomycin, 10 mM HEPES, 25 nM hRspondin, 1x B27, 5 mM

Nicotinamide, 1.25 mM N-Acetylcysteine, 500 nM A-8301, 500 nM SB202190, 50 µg/mL Primocin, 100 ng/mL hNoggin, 100 ng/mL hFGF-10, 25 ng/mL hFGF-7 (see **Table of Materials**).

NOTE: Mix gently to avoid foaming and filter through a 0.22 µm filtering system. Warm media to 37 °C within 1 h before use.

1.2. Prepare low growth factor media (LGM) as previously reported[16] without the addition of epidermal growth factor (EGF): Advanced Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture F12 (DMEM/F-12), supplemented with 1 mM HEPES, 1x Glutamine, 1x Penicillin-Streptomycin-Glutamine, 10 mM Nicotinamide, 1 mM N-Acetylcysteine, 1x B27, 500 nM A-8301, 100 ng/mL hNoggin.

NOTE: Mix gently to avoid foaming and filter through a 0.22 µm filter. Warm media to 37 °C within 1 h before use.

1.3. Thaw BME2 (Reduced Growth Factor Basement Membrane Extract, Type 2, see **Table of Materials**) on ice, at 4 °C, overnight.

2. Generating single-cell suspension and seeding of cells

2.1. Dissociate submerged BME2 organoid culture as described in the following steps (2.1.1-2.1.6).

2.1.1. Carefully aspirate media from the culture plates. Avoid touching the submerged BME2 organoid culture.

NOTE: Media Aspiration can be done as the researcher prefers, e.g., with a basic fluid aspiration system or using a pipette. Avoid touching the BME2 embedded organoids as this might result in loss of organoid biomass.

2.1.2. Trypsinize the submerged BME2 organoid culture with a suitable recombinant enzyme (see **Table of Materials**). In 6-well plate format, add 2 mL per well. Mechanically break the BME2 by repeatedly pipetting up and down. Incubate plates at 37 °C in the cell culture incubator for 5 min.

2.1.3. Transfer the suspension into a 15 mL centrifuge tube and centrifuge at 600 x *g* for 5 min at room temperature.

2.1.4. Aspirate the recombinant enzyme carefully without touching the organoid pellet.

NOTE: Residual BME2 may be present. Repeat the enzyme digestion if needed.

2.1.5. Resuspend the organoid pellet in GM (step 1.1). Add DNase I 1x 100 U/mL and incubate for 5 min at room temperature (see **Table of Materials**).

2.1.6. Centrifuge at 600 x *g* for 3 min at room temperature. Pipette off the media carefully without touching the organoid pellet and discard. Resuspend in fresh GM.

2.2. Seeding of organoid single-cell suspension

2.2.1. Pre-heat a new black, clear-bottom 96-well plate at 37 °C in the cell culture incubator for 10 min.

NOTE: Using clear bottom plates is essential to monitor organoid growth and drug response.

2.2.2. For counting, prepare a 1:5 dilution of the cell suspension in PBS (total volume: 500 µL) and count the cell suspension using a cell analyzer (see **Table of Materials**).

NOTE: Make sure to multiply by the dilution factor (x 5) to receive the final cell concentration. Alternative counting methods such as a hemocytometer can be used. Cell viability should be monitored using a viability staining assay (e.g., with Trypan Blue[17]). Using a cell analyzer, viability is assessed automatically. The viability of organoid single-cell suspensions as evaluated by the cell analyzer should be ≥95% (**Supplementary Figure 1**).

2.2.3. Calculate the volume of the cell suspension needed to seed for the experiment.

NOTE: The seeding concentration is 1500 cells/µL BME2, with 5 µL BME2 needed per well (Total number: 7500 cells/well). For one 96-well plate, 6 x 10E5 cells are required. This includes the seeding of 60 wells with organoid domes (4.5 x 10E5) and experimental surplus (calculation for a total of 80 wells).

2.2.4. Prepare one aliquot of cell suspension in a 1.5 mL microcentrifuge tube per each 96-well plate planned to be seeded if multiple 96-well plates are included in the experiment.

NOTE: Experimental surplus and separate aliquots per seeded 96-well plate are needed to account for the increased experimental bias due to handling BME2.

2.2.5. Aliquot cells from the single-cell suspension as calculated (step 2.2.3) after careful resuspension by pipetting up and down. Pellet cells at 600 x *g* for 3 min at room temperature. Carefully remove media using a P200 pipette without touching the cell pellet. Place cell pellet on ice shortly (~1 min) and resuspend the cell pellet in BME2.

NOTE: Residual media may compromise BME2 structure and rigidity. Pipette off all media carefully. Place cells on ice shortly to acclimate the cell pellet and allow cells to be resuspended in BME2 without clumping. Keep BME2 on ice constantly for it to stay in the liquid state. For one 96-well plate, 400 µL BME2 is needed for resuspension of the cell pellet. Resuspend cell pellets carefully, avoiding the introduction of bubbles.

2.2.6. Tilt your pre-warmed black, clear-bottom 96-well plate towards you. Plate cells using 5 µL of cell suspension per well and seeding cell domes at the 6 o'clock position of each well (**Figure 1A**). Fill the outer wells at the rim of the 96-well plate with PBS, and seed the cell domes in the remaining inner wells (columns 2-10 and rows B-G of a 96-well plate).

NOTE: Reverse pipetting[18] is recommended when handling BME2.

2.2.7. Do not move the 96-well plate and incubate freshly seeded cell domes in the cell culture laminar flow hood for 5 min at room temperature. Then move the plate to the cell culture incubator and incubate it for 10 min at 37 °C.

2.2.8. Carefully add 100 µL of GM per well to all the wells containing organoids (columns 2-10 and rows B-G of a 96-well plate). Add 100 µL of PBS to the outer wells at the rim of the plate.

2.2.9. Culture BME2 embedded organoids in GM media at 37 °C in the cell culture incubator for a total of 7 days. Inspect growth of organoids under the light microscope regularly.

NOTE: Please refer to **Figure 1B** and **Supplementary Table 1** for an example of expected growth progress from seeding to the day of treatment.

2.2.10. Change the media once after 3-4 days of culture: rotate the plate clockwise by 180° (organoids now at 12 o'clock position), carefully aspirate GM from opposite position to organoid dome using a multichannel apparatus if available, and then add fresh GM.

3. Drug treatment

3.1. Prepare a serial drug dilution in LGM for the drug of choice, e.g., osimertinib, to treat EGFR-mutant NSCLC organoids. Include a negative control (LGM media + 0.1% DMSO). Prepare sufficient drug aliquots of all doses according to the number of wells seeded plus experimental surplus.

NOTE: A dilution series including ≥8 doses and ranging from 1 nM-10 µM is recommended for targeted inhibitors.

3.2. Rotate the organoid plate clockwise by 180° (organoids now at 12 o'clock position). Carefully aspirate GM using a multichannel apparatus preferably.

NOTE: Avoid touching the organoid dome while aspirating the GM as this may result in loss of organoid biomass and impact results.

3.3. Add 100 µL of control (e.g., LGM media + 0.1% DMSO) or drug solution per well.

NOTE: Please refer to **Figure 1C** for the drug escalation treatment schematic in 96-well plate format.

3.4. Incubate treated organoids at 37 °C in the cell culture incubator for 5 days.

4. Readout by luminescence-based survival assay

4.1. Harvest and survival readout

4.1.1. Perform the survival assay according to Reference[19].

4.1.1.1. Thaw reagent (see **Table of Materials**) overnight at 4 °C. Equilibrate reagent in a water bath at room temperature for 30 min before use and mix by inverting.

4.1.1.2. Add an equal volume of the reagent to each well (100 µL per well). Mix thoroughly by pipetting up and down, with the pipette tip placed at the position of the organoid dome. Incubate for 5 min at room temperature in the dark.

4.1.1.3. Using a multichannel pipette, transfer approximately 75% (150 µL) of the lysate (step 4.1.1.2) to a new white, opaque-bottom 96-well plate.

NOTE: Transferring 75% of lysates to a new plate ensures the absence of bubbles in the later readout without impacting assay sensitivity.

4.1.1.4. Incubate for an additional 25 min at room temperature in the dark.

4.1.2. Record luminescence using an ELISA plate reader (integration time 0.25-1 s/per well) (see **Table of Materials**).

4.1.3. Save data in an appropriate format, e.g., a data table containing all raw reads and recording of the plate layout and drugs used.

4.2. Data analysis using statistical analysis software (see **Table of Materials**)

4.2.1. Create a new XY-table and insert data in an XY format: Rows (X) are negative control followed by escalating drug doses, with doses as log [Inhibitor] in Molar concentration. Columns (Y) are readout values that include replicates stacked along with columns.

NOTE: The concentration of the negative control should be indicated as a minimal value (given 0 is not possible in log scale), e.g., log [Inhibitor], M = -10.

4.2.2. Normalize values by selecting **Analyze** > **Normalize** and using the following parameters: normalize each subcolumn separately, Y = 0 as 0 %, “last value in each subcolumn (or first, whichever is larger)” as 100 %, results in percentages, graph the results.

4.2.3. Fit non-linear regression curve on normalized data by selecting **Analyze** > **XY analyses** > **Non-linear regression** > **Dose response - Inhibition** > **log (inhibitor) *vs*. normalized response** -- **Variable slope**.

4.2.4. Report results as a table of IC_{50} values outputted after non-linear regression analysis and graph of response curve including normalized data points as mean +/- standard deviation and fitted regression curve.

REPRESENTATIVE RESULTS:

Considerable challenges in establishing NSCLC organoids have been noted[7]. Thus, it is exciting to see recent work establishing lung cancer organoids and using them for drug treatment assays[20-22]. EGFR-mutations account for 11.3% of NSCLC cases[23]. Targeted treatment with EGFR inhibitors represents the first-line treatment option in EGFR-mutant NSCLC and has improved the overall survival and treatment safety in patients[24]. This work determined the sensitivity to the FDA-approved EGFR tyrosine kinase inhibitor osimertinib[24,25] in EGFR-mutant NSCLC organoids. EGFR-mutant NSCLC organoids were generated from surgical resection or tumor biopsy specimens of NSCLC patients and confirmed to harbor the indicated oncogenic mutation by DNA sequencing. As outlined above, EGFR-mutant NSCLC organoid models were treated with escalating doses of osimertinib and PDO viability assessed by luminescence-based cell survival readout five days after the treatment initiation. While EGFR mutant ($EGFR^{del19}$)-positive TH107 organoids showed sensitivity to osimertinib treatment with a half-maximal inhibitory concentration (IC_{50}) of 56 nM (**Figure 2A**), EGFR-mutant ($EGFR^{L858R}$)-positive TH116 organoids were resistant to osimertinib treatment with an IC_{50} of greater than 1 µM (**Figure 2B**). The sensitivity of $EGFR^{del19}$-positive TH107 NSCLC was accompanied by significant transcriptional changes, including a reduction in the expression of cell cycle-associated gene signatures and an increase in the expression of apoptosis-associated gene signatures (**Supplementary Figure 2A,B**). As a reference, response data for the sensitive $EGFR^{del19}$-positive NSCLC cell line PC9 is presented (**Figure 3A,B**). The latter includes survival analysis to escalating doses of osimertinib by a 2D luminescence-based survival assay (**Figure 3A**) and the study of signaling suppression on the level of EGFR-MAPK signaling by Western blot (**Figure 3B**). Overall, this data highlights the accuracy of the present protocol for determining drug response and distinguishing between sensitive and resistant NSCLC PDO models. Further analyses of $EGFR^{L858R}$-positive TH116 organoid and available clinical specimens are needed to determine possible resistance-associated alterations.

[Place **Figure 2** and **Figure 3** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol schematic of workflow and critical steps in the technique. (**A**) Experimental workflow including seeding of organoids in 96-well format, treatment with drug escalation at 7 days after seeding, and luminescence-based cell survival readout 5 days after treatment using an ELISA plate reader. (**B**) An example image of the $EGFR^{del19}$-positive TH107 and $EGFR^{L858R}$-positive TH330 NSCLC organoid cultures. Original cultures, cells at the time of seeding (day 0), and organoids at treatment start 7 days after seeding (day 7) are shown. Scale bar = 100 µm. Changes

in organoid diameter over the initial 7-day culture period are quantified and indicate >2-fold increase in organoid size. Relative fold changes in sizes at day 7 compared to average size at day 0 are presented below the representative images. For TH107, a fold change of 2.38 over 7 days is observed, indicating a doubling time of 5.88 days (141.12 h). For TH330, a fold change of 2.41 over 7 days is observed, indicating a doubling time of 5.81 days (139.42 h). Quantification of changes in organoid size and statistical evaluation are presented (right). Statistical significance is calculated by unpaired t-test, $p < 0.0001$. (**C**) Treatment layout for drug escalation in organoid 96-well plate format. The number of technical replicates and exemplary doses are indicated, including a negative control. The schematics are created with BioRender, a web-based illustration tool.

Figure 2: Treatment response curve of EGFR-mutant NSCLC organoid models to osimertinib escalation. (**A**) Osimertinib response in the sensitive $EGFR^{del19}$-positive TH107 NSCLC organoid model. (**B**) Osimertinib response in the resistant $EGFR^{L858R}$-positive TH116 NSCLC organoid model. Data points are presented as normalized values showing the mean +/- standard deviation, with a non-linear regression curve fitted through the data. TH107, *n* = 6 technical replicates per data point. TH116, *n* = 4 technical replicates per data point.

Figure 3: Comparative data for the treatment response to osimertinib in a sensitive EGFR-mutant NSCLC cell line and organoid models cultured in different media. (**A**) Osimertinib response in the sensitive $EGFR^{del19}$-positive NSCLC cell line PC9, determined by standard 2D-CTG assay. (**B**) Signaling suppression in the PC9 cells upon two-day treatment with osimertinib (2 µM). (**C**) Osimertinib response in the sensitive $EGFR^{L858R}$-positive TH330 NSCLC organoid model culture in LGM and GM media. (**D**) Osimertinib response in the resistant AZ021 NSCLC organoid model in LGM and GM media. Confirmation of the oncogenic $EGFR^{L858R}$ mutation in AZ021 failed and may be causative for the lack of osimertinib response. For A and C-D, data points are presented as normalized values showing the mean +/- standard deviation, with a non-linear regression curve fitted through the data. PC9, *n* = 3 technical replicates per data point. TH330, *n* = 5 technical replicates per data point. AZ021, *n* = 6 technical replicates per data point. A Wilcoxon rank test was performed on normalized data to determine the statistical significance. For TH330 (**C**), LGM *vs.* GM, ** $p = 0.0078$. For AZ021 (**D**), LGM *vs*. GM, ns $p = 0.0742$.

Supplementary Figure 1: Representative cell analyzer results for counting and viability assessment of EGFR-mutant organoid models. TH107 and TH107BC refer to different organoid models and A and B to biological replicates. For each model and biological replicate, three technical replicates are counted; all show ≥95% viability. A representative image during cell counting is presented on the right, showing robust viability and single-cell dissociation.

Supplementary Figure 2: Gene set enrichment analysis (GSEA) using bulk RNA sequencing data obtained for EGFR-mutant TH107 NSCLC organoids, comparing untreated control (DMSO) and cells treated with Osimertinib for 3 days (OSI_D3). (**A-B**) Upon targeted treatment, sensitive cells will undergo cell cycle G1 arrest and cease active proliferation. As an expression of G2M cell cycle genes is associated with active proliferation, a nominal enrichment of expression in the untreated control (DMSO) is expected. For apoptosis-related genes, a nominal enrichment in treated cells

(OSI_D3) is expected. Both have been confirmed in the EGFR-mutant TH107 NSCLC organoid model treated with Osimertinib: (**A**) GSEA for Hallmark G2M expression signature (left) shows enrichment in DMSO- treated cells. Nominal enrichment score (NES): +1.708, FDR < 0.0001. (**B**) GSEA for Hallmark Apoptosis expression signature (right) shows enrichment in Osimertinib-treated cells. NES: -1.075, FDR: ns, 0.3275.

Supplementary Figure 3: Combinatorial drug treatment in EGFR-mutant TH330 organoid model treated with Osimertinib escalation in the presence of a second additive inhibitor at a fixed concentration. Resistance-associated alterations in EGFR-mutant NSCLC, i.e., SRC and AXL activation[26-28], were pharmacological targeted by combinatorial treatment with SRC inhibitor Saracatinib (100 nM) or AXL inhibitor R428 (500 nM), *n* = 6 technical replicates per data point. Both combinatorial treatments resulted in increased treatment response, with significance for the combination of Osimertinib with SRC inhibitor Saracatinib. Statistical significance was evaluated by Wilcoxon rank test: Osimertinib versus Osimertinib + Saracatinib, **p* = 0.0195; Osimertinib *vs*. Osimertinib + R428, ns, *p* = 0.2500.

Supplementary Table 1: Change of organoid size from seeding (d0) to 7 days post-treatment (d7). (**A**) Growth development in $EGFR^{del19}$-positive NSCLC organoid TH107. (**B**) Growth development in $EGFR^{L858R}$-positive NSCLC organoid TH330.

DISCUSSION:

This manuscript develops and describes a standardized protocol for assessing drug sensitivity in NSCLC-derived 3D PDO models. In addition to drug sensitivity studies, further characterization of available organoid models is needed to determine the underlying causes for differences in drug sensitivity. This may include genetic profiling of organoids and patient specimens and other analysis available for organoids, such as immunohistochemistry staining for differentiation markers and general cellular signaling biomarkers and physiology[13,29].

Critical steps in the protocol

The protocol outlined herein provides a standardized workflow that allows accurate and reproducible drug sensitivity analyses when followed carefully. Particular care should be taken in the following steps: TrypLE and DNAse I digestion during generation of single-cell suspensions, seeding of single-cell suspensions in BME2, monitoring organoid growth until treatment, media changes, and disruption and lysis of BME2 embedded organoids during luminescence-based cell survival readout. (1) While additional DNAse I digestion after TrypLE-based dissociation of organoids is not essential for expanding organoid models during regular culture maintenance, DNAse I digestion should not be omitted when seeding for drug escalation experiments as it ensures better separation of organoid clusters into single-cell suspensions and accurate cell counting. (2) Seeding of single-cell suspension in BME2 represents a critical step given the solidification of BME2 at room temperature. Thus, a maximum of 1-2 rows needs to be seeded at once, and samples should be placed on ice before additional rows are seeded. Of note, cells need to be pipetted up and down when seeding is continued to allow for a homogeneous cell suspension. (3) Organoid growth needs to be monitored carefully during the 7-day expansion from seeding to treatment. An example of the expected development is given in **Figure 1B** and

Supplementary Table 1. Of note, assessing changes in organoid size by brightfield microscopy and image analysis as presented in **Figure 1B** may allow for a precise evaluation of differences in organoid growth and doubling times. Doubling times can have an impact on drug responses, as recently discussed in the literature[30]. If the organoid growth rate exceeds the presented example significantly, a shorter expansion time until the start of treatment and shorter treatment duration can be considered. (4) In addition, special care should be taken when changing media to avoid aspirating organoids. The seeding position of BME2 embedded organoids at the 6 o'clock position allows for a safe aspiration of media when plates are turned clockwise by 180° and media is aspirated at the opposite position of the organoids. (5) Finally, thorough lysis of BME2 embedded organoids during the survival readout is essential to record accurate results. According to the manufacturer's instructions, samples should be pipetted up and down repeatedly, ideally using unfiltered tips, to ensure proper lysis. Incubation times should be followed as described. Further, transferring 75% of the lysate (instead of the total volume) to a white, opaque-bottom 96-well plate for the final readout using an ELISA plate reader allows for an appropriate assessment, as this assures the same volume in each well and the absence of air bubbles that can be introduced by vigorous pipetting.

Of note, profiling of drug responses in BME2-embedded organoid cultures may show a higher standard deviation than observed in regular cell line cultures (**Figure 2, Figure 3A**). The higher standard deviation is based on several factors, including an increased likelihood of minor variations in seeding when working with BME2 and differences in individual organoid growth rates across wells over the initial 7-day growth period. Thus, equal or more than four technical replicates per drug concentration should be seeded.

Most importantly, the presence of malignant cells carrying the oncogenic driver mutation and limited contamination by normal airway epithelial cells must be carefully evaluated. Challenges in NSCLC establishment can favor the outgrowth of normal airway epithelial cells[7]. Copy number profiling or PCR- and sequencing-based approaches to confirm the presence of the oncogenic driver mutations are the methods of choice to ensure the quality of NSCLC organoid cultures.

Modifications and troubleshooting of the method

Media and respective growth factors added to basic media solutions can significantly impact drug response to targeted inhibitors. They activate bypass receptors and signaling pathways that influence and limit drug response (e.g., FGF, HGF, EGF)[26]. While a growth-factor rich and tailored media may be optimal for expanding organoid culture, drug escalation and sensitivity assessments should be performed in a reduced growth-factor media, as outlined above. This is based on internal experience comparing different media formulations and drug response data (**Figure 3C**). While media solutions can affect the degree of sensitivity to certain drug treatment and can shift IC_{50} values, robust phenotypes of sensitivity or resistance are apparent irrespective of media formulation (**Figure 3C,D**). In addition, general consistency in the media formulation and profiling drug responses across organoid cultures is recommended, and an equal or more than four technical replicates per concentration needs to be seeded. This is particularly important to benchmark ranges in sensitivity *vs*. resistance for the Inhibitor of interest.

Limitations of the method

The protocol presented here describes the sensitivity of NSCLC 3D cancer organoid models to targeted inhibitors when patient-derived cancer cells are cultured. Additional experiments, including pharmacodynamic analysis regarding pathway inhibition and sequencing analysis for the presence of the driver oncogene and secondary mutations, are needed for a detailed characterization of drug resistance and sensitivity. Further, bystander factors such as microenvironmental stimuli derived from interactions or secreted factors by non-cancer bystander cells in the tumor microenvironment are not accounted for, and novel protocols are needed when co-culture organoid models with immune or stromal cells are attempted. Recent work has highlighted the use of organoid models to recapitulate tumor microenvironment interactions and profile responses to immune checkpoint inhibitors, such as anti-PD-L1 treatment[13,31].

The significance of the method with respect to existing / alternative methods

3D cancer organoid models recapitulate the genetic diversity and determinants of treatment response present in the original tumor[4-6]. Notably, spatial and temporal heterogeneity can promote tumor evolution, and parallel emergence and the sequential development of tumor subclones can occur[32,33]. Intratumor heterogeneity is significant for the selection of more resilient tumor cells under therapeutic pressure[9,34,35]. The protocol provided here allows for a rapid assessment of sensitivities to treatment with targeted inhibitors in patient-proximate samples. Thus, organoid models have advantages over more conventional homogeneous cell line models lacking genetic diversity or long-term studies using cell lines or patient-derived xenografts. Further, the present protocol allows scaling up to multiple arms of treatment and combinational treatment approaches with few limitations regarding cost and analytic capacity. As such, adding a second drug of interest at a fixed dose while escalating the primarily targeted Inhibitor and comparing it to the escalation of the primarily targeted Inhibitor alone allows for efficiently evaluating the potential combinatorial effects and with minimal additional biomass required (**Supplementary Figure 3**). Compared to imaging-based assessments used to monitor organoid development and drug response, the luminescence-based cell survival assay described here has similar sensitivity with minimal equipment and training required.

Importance and potential applications of the method in specific research areas

Developing a standardized pipeline that allows for establishing cancer organoid models from patient specimens and the subsequent drug sensitivities profiling holds significant clinical applicability potential. *Ex vivo* pharmacological profiling has gained recognition in detecting vulnerabilities and resistant-associated features in tumors, correlating to treatment response in patients[36,37]. Significantly, *ex vivo* profiling of drug sensitivities may aid in treatment selection in the clinic and the design of rational combinational treatments addressing resistance mechanisms. Overall, this approach could help to enable improved personalized strategies for molecular therapy or combinatorial treatment regimens. The latter may help target drug tolerance and resistance mechanisms early and deepen clinical response to improve patient outcomes in the future.

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Figure 1

Click here to access/download;Figure;Fig1-R2.pdf

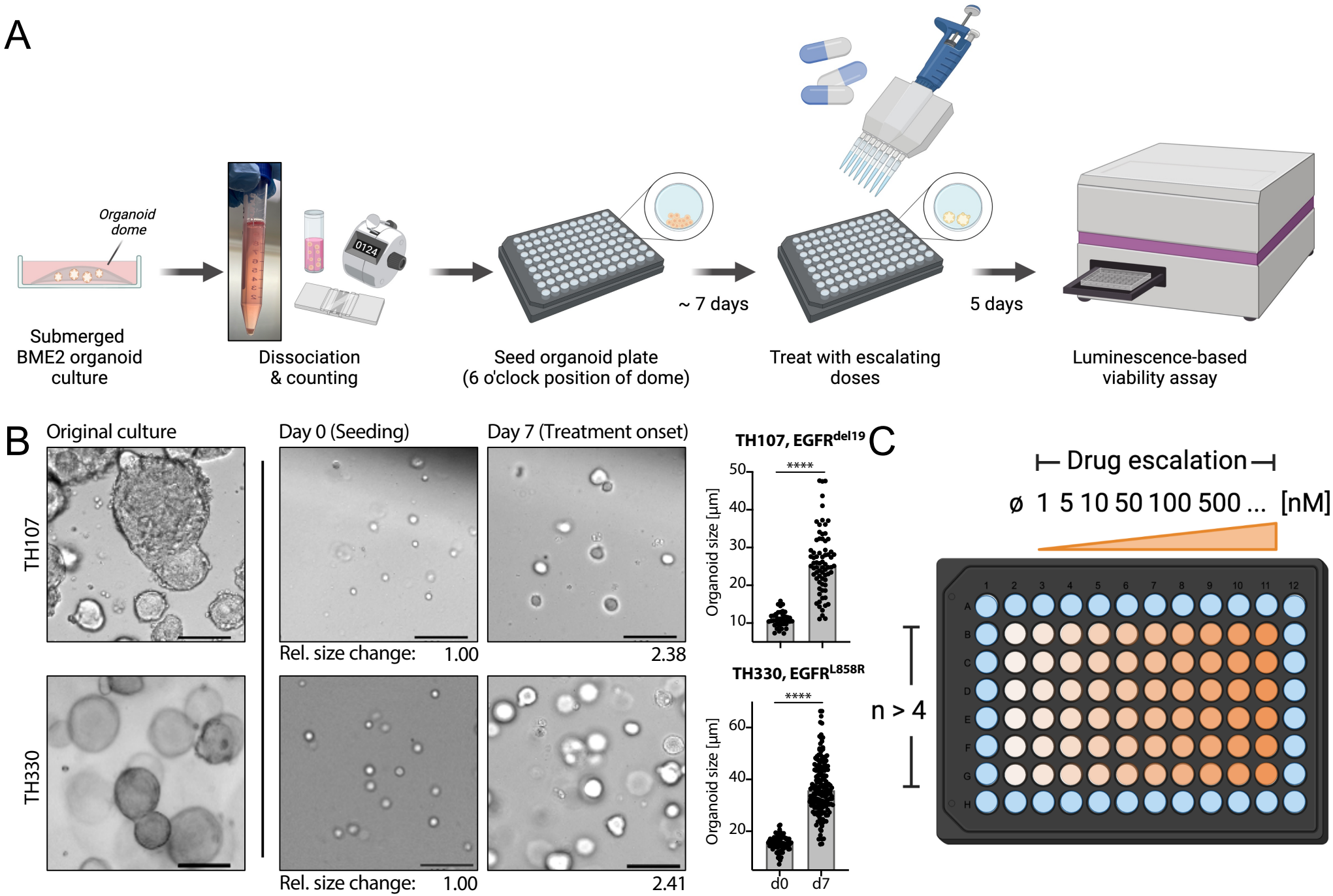


Figure 2

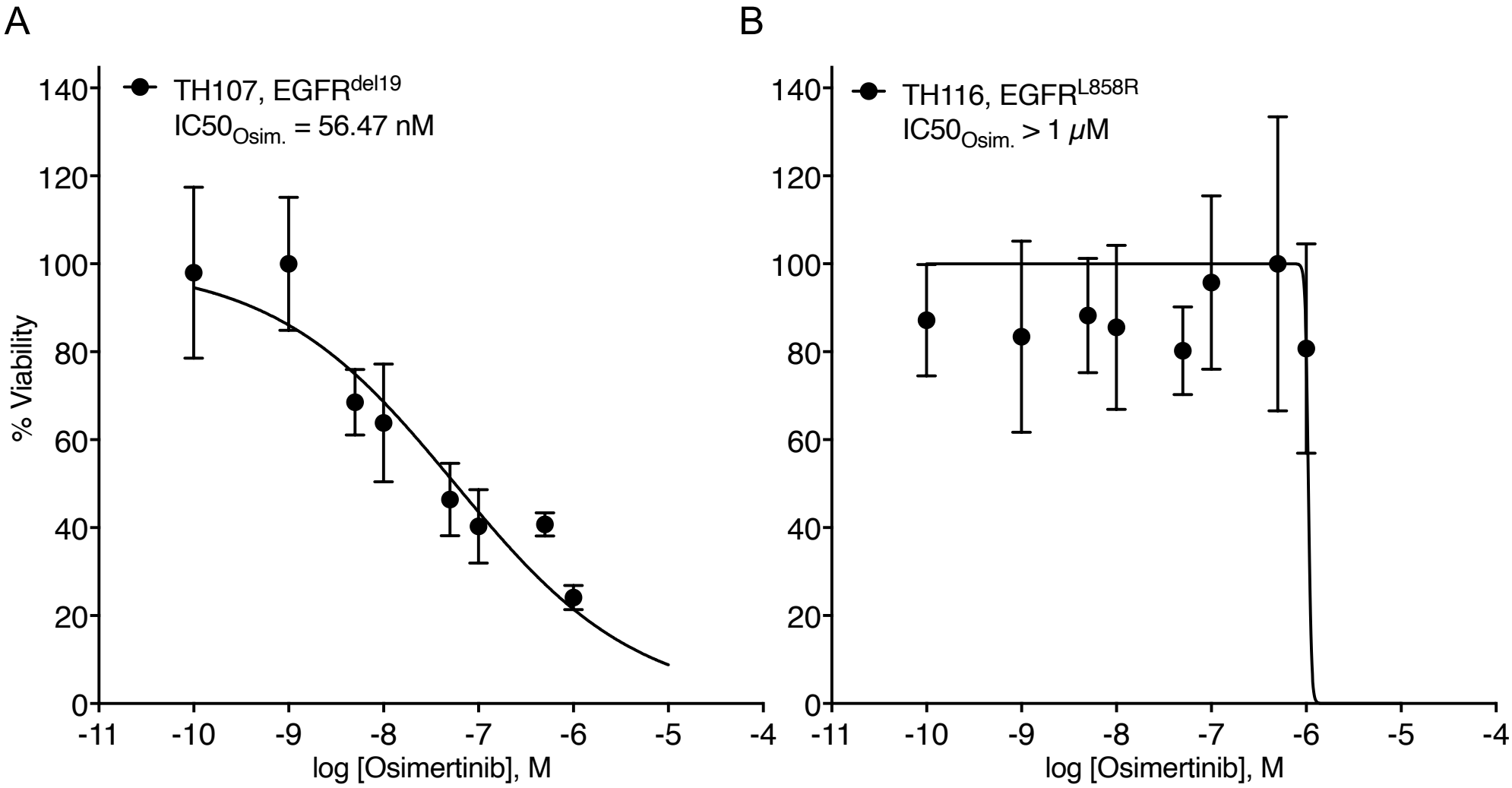
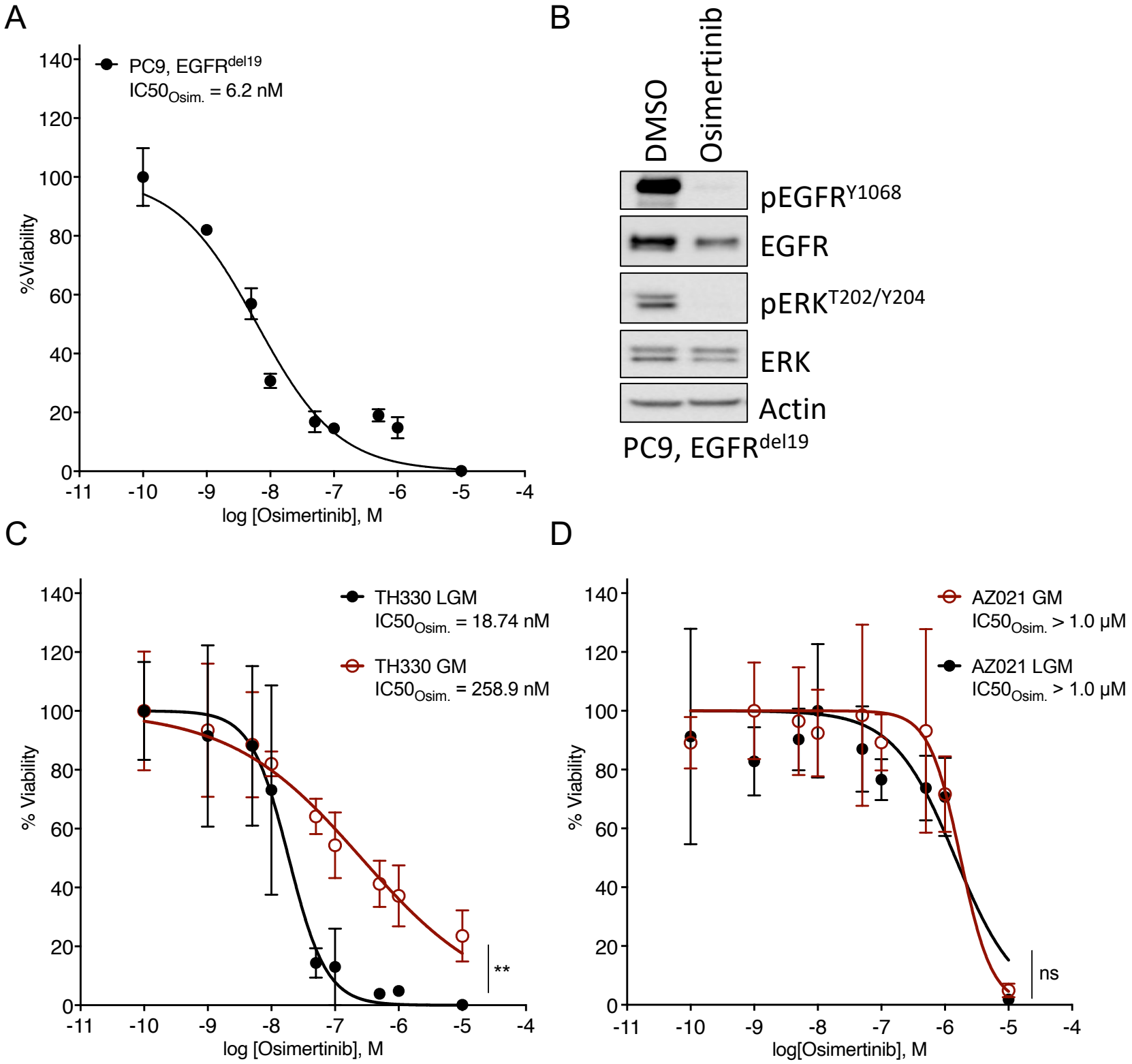


Figure 3



Authors response to Editorial and Reviewer comments

Thank you very much to the Editor and Reviewers for their helpful suggestions and detailed feedback on our manuscript. We have addressed the points as outlined below in our point-by-point response.

In brief, we have now:

- added the representative Vi-Cell XR Cell Analyzer results as Supplementary Figure 1, the gene set enrichment analysis for RNA sequencing data of EGFR-mutant organoids as Supplementary Figure 2, the combinatorial treatment data as Supplementary Figure 3, and the tabular data on changes in the organoid sizes as Supplementary Table 1;
- addressed additional comments raised by the editor directly in the text; and
- highlighted the need for the selection and confirmation of cancer cell composition in NSCLC organoids as outlined by Reviewer 3.

Editorial comments:

Author response: We sincerely thank the Editor for the careful review of our manuscript. The points raised have been addressed and we believe the manuscript is improved as a result.

1. Please include the representative Vi-Cell XR Cell Analyzer results for counting and viability assessment (provided in response to Major concern 2 of Reviewer 1) as a Supplementary Figure. Please reference this in the manuscript text and provide a legend in the Figure and Table legend section.
Author response: We have added the representative Vi-Cell XR Cell Analyzer results as Supplementary Figure 1. Supplementary Figure 1 is referenced in the note to step 2.2.2 .

2. Please include the tabular data on changes in the organoid sizes (provided in response to the Major concern of Reviewer 2) as a Supplementary Table. Please reference this in the manuscript text and provide a legend in the Figure and Table legend section.
Author response: We have added the tabular data on changes in the organoid sizes as Supplementary Table 1. Supplementary Table 1 is referenced in step 2.2.9 and in the discussion (Critical step in the protocol, p.9, line 438).

3. Please include the GSEA using bulk RNA sequencing data obtain for EGFR-mutants (provided in response to Major concern of Reviewer 2) as a Supplementary Figure. Please reference this in the manuscript text and provide a legend in the Figure and Table legend section.
Author response: We have added the GSEA using bulk RNA sequencing data obtain for EGFR-mutant organoids as Supplementary Figure 2. Supplementary Figure 2 is referenced in the representative results section.

4. Please include the Figure on the combinatorial treatment approaches (provided in response to Major concern of Reviewer 2) as a Supplementary Figure. Please reference this in the manuscript

text and provide a legend in the Figure and Table legend section.
Author response: We have added a figure on combinatorial treatment approaches as Supplementary Figure 3. Supplementary Figure 3 is referenced in the discussion (The significance of the method with respect to existing / alternative methods, p.11, line 519). Of note, we were not able to use the drug combinations as presented in the editorial response of our first revision, as this data is included in an independent manuscript. Thus, we have prepared new data using the same approach and presenting clinically relevant combinations with SRC inhibitor Saracatinib and AXL inhibitor R428. We have linked to literature highlighting the relevance of SRC and AXL in EGFR inhibitor resistance in NSCLC.

5. Additional comments are in the attached manuscript.
Author response: We have addressed all comments raised in the manuscript and added a short response in each comment. We would like to thank the Editor for all comments raised.

Reviewers' comments:

Reviewer #3:

Rabago et al. provide a brief protocol which enables assessment of proliferation of NSCLC organoids with small molecules in 96-well format. Although relatively straightforward, I found the practical tips/tricks within the article useful and I think there is sufficient detail for replication. The authors have made appropriate edits based on the two previous reviews and I believe the manuscript will represent a valuable addition to the literature in this area.

Author response: We sincerely thank the Reviewer for the careful review of our manuscript.

* I agree with the previous reviewers that inclusion of an establishment protocol(s) would have improved the manuscript's reach since this is a major barrier to laboratories using NSCLC organoids.
Author response: The establishment of lung cancer organoids from de-identified clinical specimens is performed in close collaboration with organoid centers across UCSF and Stanford. During the initial revision, we have added a short paragraph at the beginning to reflect this and to link to relevant literature. In addition, we provide the detailed composition of our culture media, which enables the outgrowth and maintenance of NSCLC organoids. We hope that readers benefit from having this information. Further, we included a reference to a publication from the Clevers lab that addresses challenges in the establishment of NSCLC organoids (introduction and representative results section, Ref. 7), and we highlighted the need for the confirmation of oncogenic drivers / presence of malignant cells by DNA sequencing in our models.

* A key QC step worthy of further mention is the need to select for cancer cells from normal airway epithelial cells; as has been shown in organoid and 2D protocols, I fear many groups will end up testing their inhibitors on airway basal cell cultures rather than NSCLC cells in these assays.
Author response: We agree that it is of utmost importance to confirm the presence of malignant cells within the organoid culture as well as the oncogenic driver in order to profile drug responses accurately. We have addressed this in more detail in the result section - highlighting that the organoid cultures were sequenced, and the oncogenic driver mutation was confirmed in both sensitive and resistant organoid cultures. Further, we have added one additional paragraph in the discussion (Critical steps in the protocol, p.10, line 472-476) to highlight this point.

* 2.2.5. - clarify that this is tilting the plate towards you? It also wasn't immediately obvious to me what "reverse pipetting" is.
Author response: We have clarified that plates have to be tilted towards the operator (step 2.2.6, p.5, line 185). We have also added a reference that explains differences of forward and reverse pipetting (step 2.2.6, p.5, line 190).

Reviewer #4:
Manuscript Summary:
Rabago et al. describe a detailed protocol to perform pharmacological studies with molecular therapy in lung cancer PDO, all previously stabilized by other groups. They propose a 96-well based format using CTG assay. The revised ms is improved and the data are convincing. The procedure is useful for the scientific community.

Author response: We sincerely thank the Reviewer for the careful review of our manuscript.

Concerns:
The authors have addressed all the major and minor concerns of the previous reviewers. I agree that the protocol for establishing PDO is not new and that the CTG assay is not so innovative but the procedure is well-describe and I am sure is reproducible in any lab. I personally prefer this king of approach rather than very innovative protocol not so robust and reproducible.

In my opinion the ms is now suitable for publication in JOVE.

Authors response to Editorial and Reviewer comments

Thank you very much to the Editor and Reviewers for their helpful suggestions and detailed feedback on our manuscript. We have addressed the points as outlined below in our point-by-point response.

In brief, we have now:

- Updated our title to reflect the focus of the protocol on drug sensitivity profiling in EGFR-mutant NSCLC organoids
- Included a paragraph regarding organoid establishment linking to previously published work and methods we are using in collaboration with research partners (page 3, line 87-90)
- Added a note regarding viability assessment of organoids during seeding (page 4, line 161-163)
- Presented new quantitative data for the assessment of organoid size, a comparison to a second organoid model and images of original cultures prior to dissociation and seeding in Figure 1B
- Expanded our discussion regarding organoid size assessment and doubling time (page 10, line 427-434), increased standard deviations when assessing drug response in organoid models (page 10, line 446-451), methods to profile combinatorial treatments (page 11, line 509-512)
- Removed commercial terminology and corrected the wording for matrigel to BME2 across the manuscript

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author response: We have thoroughly proofread the manuscript. To the best of our knowledge, there are no spelling or grammatical errors.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials (including reagents, instruments, software, etc.). Please sort the Materials Table alphabetically by the name of the material

Author response: This has been corrected. Thank you.

3. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Author response: We hope the protocol provides enough detail to be replicated easily by readers.

4. Please add more details to your protocol steps:

Step 1.3/2.2.2/4.1/4.2: Please remove the commercial term.
Author response: This has been done and corrected. Thank you.

Step 2.1: This step mentions the organoid culture dissociation. In which the culture was prepared and how? Please clarify.
Author response: Establishment of organoid culture: We are using de-identified clinical specimens obtained on IRB-approved clinical protocols for PDO generation. The establishment of lung cancer organoids from these clinical specimens is performed in close collaboration with organoid centers at UCSF (Organoid D2B unit lead by J.P. Roose) and Stanford (C.J. Kuo lab). Protocols regarding the preparation of organoids from clinical specimens across centers vary and prior publications describe the processing of healthy lung or lung cancer specimens into organoids. All laboratories have individual research interests working on organoids. When organoids are stably established, we receive a passage of live culture that we maintain and use for drug escalation experiments, etc. We have included a short paragraph at the beginning of the protocol to address the contribution from the different teams for the use of clinical specimens to generate organoid cultures. (page 3, line 87-90)

Dissociation of organoids: Dissociation of organoids into single cell suspensions is based on TrypLE-based digestions and an additional incubation step in DNAse I before counting and seeding cells. The additional DNAse I step reduces clumping and ensures a high quality of the single cell suspension as well as accurate counting, which is essential for limiting variation in seeded cell numbers per well and later viability readout. We have updated text paragraphs in the discussion (section "*Critical steps in the protocol*"), which are focused on the necessity of additional DNAse I digestion after TrypLE-based dissociation. (page 9, line 408-415)

Step 2.1.2: Could you please specify what is meant by Matrigel dome?
Author response: We have rephrased this term in the text and changed the schematic in Figure 1A to clarify. The matrigel dome is the submerged BME2 organoid culture, which is seeded in 3D "dome"-structures that form when the BME2 solidifies. Figure 1A now clearly indicates the dome-like structure of submerged organoids as the starter culture.

Step 2.1.3: Replace "Falcon" tube with "centrifuge" tube.
Author response: This has been corrected. Thank you.

Step 3.2: How the rotation is done?
Author response: We provided additional information to allow for a better understanding by the reader: "Rotate the organoid plate clockwise by 180 ° (organoid domes now at 12 o'clock position)." (page 6, line 231-232 and 242)

5. Figure 2: Please include a space between "log" and "[Osimertinib]' in log[Osimertinib].
Author response: This has been corrected. Thank you.

6. This manuscript has only two Figures. The Supplementary Figure can be included as one of the main Figures.
Author response: Supplementary Figure 1 is now included as Figure 3.

7. Please spell out the journal titles in the References.
Author response: This has been corrected. Thank you.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the current manuscript, the authors describe a detailed protocol for testing NSCLC PDOs' sensitivities to EGFR targeted agents using a cell culture plate-based assessment. The authors also tested the presence of growth factors in the medium on drug sensitivities. However, the protocol presented in this study for seeding organoids and drug treatment did not show much differences with previous studies. In addition, the author may need to check the spelling and grammar throughput the manuscript.

Author response: We sincerely thank the Reviewer for the careful review of our manuscript. The points raised have been addressed and we believe the manuscript is improved as a result.

Major Concerns:

1. The authors provide a step-by-step protocol for digesting and seeding lung tumor organoids for drug screens, I believe that a brief description of the steps for deriving lung cancer organoids from clinical samples should be included.

Author response: The establishment of lung cancer organoids from de-identified clinical specimens is performed in close collaboration with organoid centers at UCSF (Organoid D2B unit lead by J.P. Roose) and Stanford (C.J. Kuo lab). Establishment protocols across centers vary and prior publications describe the processing of specimens from normal lung or lung cancer specimens. We have included a short paragraph at the beginning of the protocol to address the contribution the different teams for the use of clinical specimens, with respective literature references. (page 3, line 87-90) Further, we have rephrased our title to highlight the focus on the profiling of drug sensitivities in our protocol, i.e. “Profiling sensitivity to targeted therapies in EGFR-mutant NSCLC patient-derived organoids”.

2. It is very interesting that the authors used DNase instead of TrypLE™ Express to digest organoids into single cells as this method was rarely adopted. Did the author compare the differences of these two reagents on the viability of lung cancer organoids?

Author response: As outlined in protocol step 2, dissociation of organoids into single cell suspensions is based on TrypLE-based digestions and an additional incubation step in DNAse I before counting and seeding cells. We have updated text paragraphs in the discussion (section “*Critical steps in the protocol*“), which are focused on the necessity of additional DNAse I digestion after TrypLE-based dissociation. We hope that rephrasing will provide more clarity regarding this point. (page 9, line 408-415) Of note, our prior experience with TrypLE alone showed increased cell clumping and less accuracy in counting and seeding. Thus, we advise for the additional DNAse I digestion after TrypLE-based dissociation.

The viability of the organoid single cell suspension after TrypLE and DNAse I digestion is ≥ 95%, as measured by the Vi-Cell XR Cell Analyzer before plating. This assessment is done automatically by the cell analyzer in the counting process. Further, no significant number of dead cells is visible as monitored by brightfield microscopy at the day of seeding or the initial 7-day growth period (Figure 1B). The need to monitor organoid growth and fitness is explained in

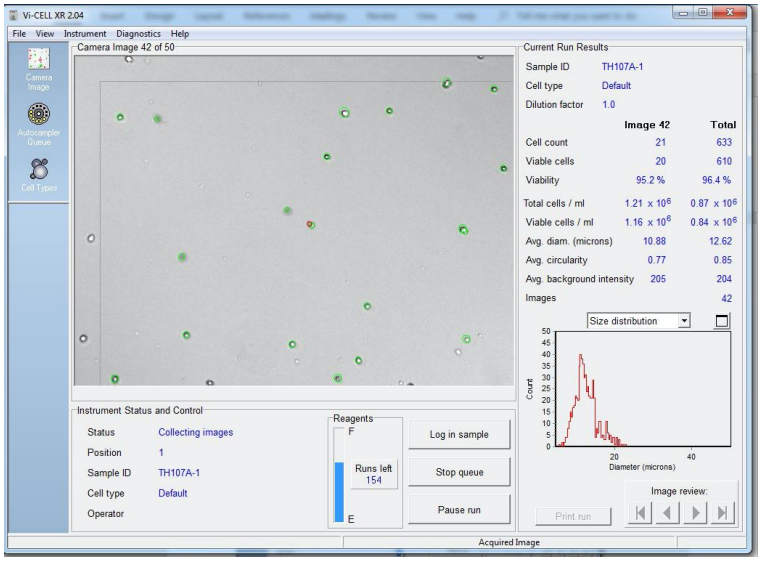
the discussion, section "*Critical steps in the protocol*" (point iii). Further, we have added a NOTE at the counting step to indicate the need for viability assessment. (page 4, line 161-163)

As an example, we have presented representative Vi-Cell XR Cell Analyzer results for counting and viability assessment below. TH107 and TH107BC refer to different organoid models and A and B to biological replicates. For each model and biological replicate, three technical replicates are counted. All show ≥ 95% viability. A representative image during cell counting is presented on the right, showing robust viability and single cell dissociation.

Vi-CELL XR 2.04

Beckman Coulter, Inc.

Sample ID	Viability (%)	Total cells /ml (x10^6)	Viable cells /ml (x10^6)
TH107A-1	96.4	0.87	0.84
TH107A-2	96.5	0.92	0.88
TH107A-3	96.6	0.82	0.80
TH107B-1	96.2	0.85	0.82
TH107B-2	97.2	0.86	0.83
TH107B-3	95.8	0.80	0.77
TH107BC-A-1	94.6	1.08	1.02
TH107BC-A-2	94.3	1.05	0.99
TH107BC-A-3	96.1	1.00	0.96
TH107BC-B-1	95.8	1.13	1.09
TH107BC-B-2	95.8	1.15	1.10
TH107BC-B-3	94.8	1.10	1.04



Cell count and viability of organoid cells after TrypLE + DNAse I-mediated digestion as measured by a ViCell XR Cell Analyzer. All samples are counted in technical triplicates.

"DNAse" should be "DNase I"?

Author response: Correct. This has been changed to DNAse I in the text and material table.

In addition, bright-field images of organoids under light microscope after key digestion steps should be helpful, e.g., before and after DNase I digestion.

Author response: Representative images of organoid cells are presented in Figure 1B. We have now included images of original cultures prior to TrypLE + DNAse I-mediated dissociation, in addition to images of freshly seeded cells.

3. Figure 1B, the picture of organoids is not clear and the size of organoids seems too small. "IC50" ->"IC50" throughput the manuscript.

Author response: In Figure 1B, representative images of cells at the time of seeding (day 0) and at treatment initiation (day 7) are shown. We have now added the comparison to the original culture with fully grown organoids. Of note, treatment initiation at an early organoid state is necessary given several factors: comparability across organoid models even upon minor changes in size or doubling time, best response to drug at a lower cellularity state, and clear distinguishment of sensitive and resistant cultures (Figure 2). We have added representative images for a second organoid model (TH330) in Figure 1B as well as the quantification of changes in organoid size.

4. Figure 2 and Supplementary Figure 1, did the replicates refer to technical replicates or biological replicates?
Author response: Replicates refer to technical replicates. The information has now been included across figures legends for Figure 1, Figure 2 and Supplementary Figure 1 [now Figure 3] as well as in the discussion.

Also, the error bars seem too large, could the author explain the inconsistent results between these replicates?
Author response: Profiling of drug responses in BME2-embedded organoid cultures shows a higher standard deviation than observed in regular cell line cultures. The higher standard deviation is likely based on several factors, including a higher likelihood of minor deviations in seeding when working with BME2 and differences in individual organoid growth rates over the initial 7-day growth period. Thus, equal or more than 4 technical replicates per drug concentration should be seeded, as outlined in the discussion (section "*Modifications and troubleshooting of the methods*"). In order to clarify this point, we have added an additional paragraph in the discussion (section "*Critical steps in the protocol*"). (page 10, line 446-451)

5. Supplementary Figure 1C, the author may need to calculate the statistical difference between the two treatments.

Author response: The statistical significance for the difference in treatment response between the two media formulations has now been calculated for Supplementary Figures 1C and 1D [now Figure 3C and D]. *P* values are indicated in the individual figures and the figure legend.

6. Step 2.1.3, did the author inactivate TrypLE before the following centrifuge step? I am asking this because TrypPLE may reduce cell viability.
Author response: According to the manufacturer's instruction, dilution alone inactivates TrypLE and no further FBS-based or similar inactivation is needed (https://www.thermofisher.com/order/catalog/product/12604013). Pelleting cells after TrypLE digestion and resuspension in GM is necessary to inactivate TrypLE. Incubation and centrifugation times are optimized to allow the dissociation of the BME2 matrix while maintaining cell viability. The viability of the organoid single cell suspension is ≥ 95%, as reported by the Vi-Cell XR Cell Analyzer during counting (now indicated at page 4, line 161-163). Further, no significant number of dead cells is visible as monitored by brightfield microscopy at the day of seeding or the initial 7-day growth period (Figure 1B).

7. Step 2.2.6, why did the authors keep the culture plate in the culture hood for 5 min? From my experience, small volumes of Matrigel seeded in culture plate are not that fragile.
Author response: We allow the BME2 to solidify in the culture hood before moving it, irrespective of volume. While this may be more critical for larger volumes to form a BME2 dome, we are reasonably confident that even in small volumes a stronger 3D structure of the BME2 is maintained and the "spreading" of BME2 along the well border is reduced. Given that we use a defined 6 o'clock position for seeding the BME2-cell suspension and for performing later lysis with 3D-CTG reagent by pipetting up and down at the identical position, we think that this step is critical and have not modified step 2.2.6.

Minor Concerns:
1. Table of Materials. "for both medias" ->"for both media", media is plural.

Author response: This has been corrected. Thank you.

2. Line 66. "therapies targeted against residual tumor cells" ->" therapies targeting residual tumor cells".
Author response: This has been corrected. Thank you.

3. Line 85. Regarding the basal medium, the authors may refer to advanced DMEM/F12, as the components of advanced DMEM/F12 are different and using DMEM/F12 as the basal medium will affect the growth of tumor organoids in practice.
"Ham's nutrient mixture F12 (DMEM/F-12) GlutaMAX supplement" -> "supplemented with 1x GlutaMAX".
Author response: We have indicated the components of both media, GM and LGM, accurately with the respective names as indicated by the manufacturers. Changing nomenclature may affect the clarity of the information and result in mismatches with the materials listed in the supplementary table of materials and providers. We agree that growth kinetics in GM and LGM differ given the differences in media formulation and added growth factors. Testing different media, we have selected GM for improved organoid outgrowth and maintenance of cultures or growth periods after seeding for drug assays. LGM is better suited for drug escalation assays, as the most consistent and accurate drug responses have been observed with IC50 values of sensitive organoid lines correlating with reference values for sensitive cell lines (Figure 2 and Supplementary Figure 1 [now Figure 3]).

4. The authors may need to place a space between numbers and abbreviated units, e.g., Line 96-97.
Author response: This has been corrected. Thank you.

5. Line 97. "100 μg/mL hNoggin", I believe this is a typo, 100 μg/mL is a very high concentration.
Author response: This has been corrected. Thank you.

6. Line 102. "Thaw matrigel (Cultrex RGF Basement Membrane Extract, Type 2)", Both Matrigel and BME2 are commonly used for the culture of organoids but they are different.
Author response: This has been corrected. Thank you.

7. Line 267. "at 5 days after treatment" ->"5 days after treatment"
Author response: This has been corrected. Thank you.

8. Line 299. "genetic profiling of organoid" ->"genetic profiling of organoids"
Author response: This has been corrected. Thank you.

9. Line 370. The authors claimed that their plate-based 3D-CTG assay had similar sensitivity to imaging-based assessment of drug responses. To my knowledge, the most widely used method for quantifying drug response is luminescence-based measurement.
Author response: We agree that luminescence-based protocols are widely used. We have now indicated this technical term in the respective sentence for accuracy. As this section in the discussion focuses on alternative methods, we indicated image-based readouts and relevant literature on monitoring drug responses by imaging-based assays (e.g. PMID: 29180611).

Reviewer #2:

Manuscript Summary:

Rabago et al. attempted to develop a standardized protocol for screening organoids models derived from NSCLC tissues. The protocol is based on a 96 well format screening. The organoids are dissociated, plated on 96 well plates embedded in Matrigel, and overlayed with LGM. The organoids are left for 7 days to form prior to drugging. The drugs are left on the plates for 5 days after which the viability is measured using CTG assay. Data is analyzed using GraphPad Prism software. The representative data to illustrate the effectiveness of this protocol was based on testing Osimertinib on an osi-sensitive and osi-resistant NSCLC organoid model.

Author response: We sincerely thank the Reviewer for the careful review of our manuscript. The points raised have been addressed and we believe the manuscript is improved as a result.

Major Concerns:

The protocol is not novel and lacks significant details:

Author response: We apologize for the lack of clarity regarding our main focus. Our aim is to profile drug sensitivity in NSCLC cancer organoids with enough detail to allow for reproducibility by any reader, while we reference establishment protocols that indeed are focus of current literature. We have adapted our title to reflect this.

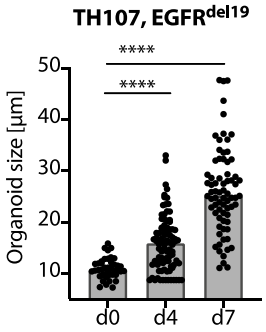
Considerable challenges in establishing NSCLC organoids have been noted by Clevers et al., with a successful establishment rate of only 17% for lung cancer organoids (PMID: 32375033). Thus, it is exciting to see a limited number of publications that have succeeded in establishing lung cancer organoids and using them for drug treatment assays (PMIDs: 31488816, 33972544, 31694835). Hu et al. (PMID: 33972544) present an elegant integrated superhydrophobic microwell array chip (InSMAR-chip)-based assay in lung cancer organoids with an AlamarBlue-based viability readout 72 hours after treatment initiation. While sufficient detail regarding treatment and readout is provided, access to such specialized techniques is limited. On the other hand, other publications regarding NSCLC organoids that use luminescence-based readouts (PMIDs: 31488816, 31694835) often lack detail regarding organoid handling, seeding and readout for drug sensitivity assays. Further, none of the indicated publications has presented data on the drug response to the EGFR inhibitor osimertinib, which is now approved as frontline therapy in EGFR-mutant NSCLC. We hope to fill this gap and provide a detailed protocol for a luminescence-based cell viability readout for NSCLC organoids – in general an emerging and evolving and challenging area of research.

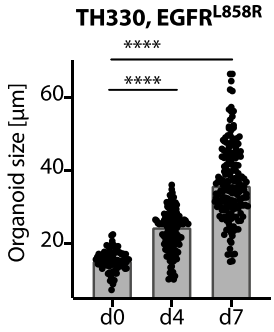
- Are the NSCLC organoids established or newly initiated from tissues?

Author response: Please also see Reviewer 1, comment 1. The establishment of lung cancer organoids from de-identified clinical specimens is performed in close collaboration with organoid centers at UCSF (Organoid D2B unit lead by J.P. Roose) and Stanford (C.J. Kuo lab). Establishment protocols across centers vary and prior publications describe the processing of specimens from normal lung or lung cancer specimens. We have included a short paragraph at the beginning of the protocol to address the contribution the different teams for the use of

clinical specimens, with respective literature references (page 3, line 87-90). Further, we have rephrased our title to highlight the focus on the profiling of drug sensitivities in our protocol, i.e. “Profiling sensitivity to targeted therapies in EGFR-mutant NSCLC patient-derived organoids”.

- What are the doubling rates of NSCLC organoids? More representative images of models used in this study are needed at different magnification to assess the status of organoids.
Author response: In Figure 1B, representative images of cells at the time of seeding (day 0) and at treatment initiation (day 7) are shown. We have now added representative images for a second organoid model (TH330) as well as the quantification of changes in organoid size. Further, we have included the comparison to the original culture with fully grown organoids. During the initial 7-day growth period, organoids double in size as presented in Figure 1B and again here with an additional quantification at day 4. Relative fold changes in sizes at day 7 compared to average size at day 0 are presented: For TH107, a fold change of 2.38 over 7 days is observed, indicating a doubling time of 5.88 days (141.12 hours). For TH330, a fold change of 2.41 over 7 days is observed, indicating a doubling time of 5.809 days (139.42 hours).





	Transpose of Organoid-growth_TH107		
	average size [µm]	SD	N
d0	11.00	2.00	41
d4	15.89	5.40	90
d7	26.13	8.32	73
	Rel fold change		
d0	1.00		
d4	1.45		
d7	2.38		

	Transpose of Organoid-growth_TH330		
	average size [µm]	SD	N
d0	15.32	2.81	90
d4	23.26	5.71	134
d7	36.96	10.26	172
	Rel fold change		
d0	1.00		
d4	1.52		
d7	2.41		

Changes in organoid size during the initial 7-day growth period prior to treatment initiation, with the average size in each condition and relative fold changes compared to day 0 indicated in the table below.

Of note, treatment initiation at an early organoid state is necessary given several factors: comparability across organoid models even upon minor changes in size or doubling time as well as best response to drug at a lower cellularity state and clear distinguishment of sensitive and resistant cultures (Figure 2).

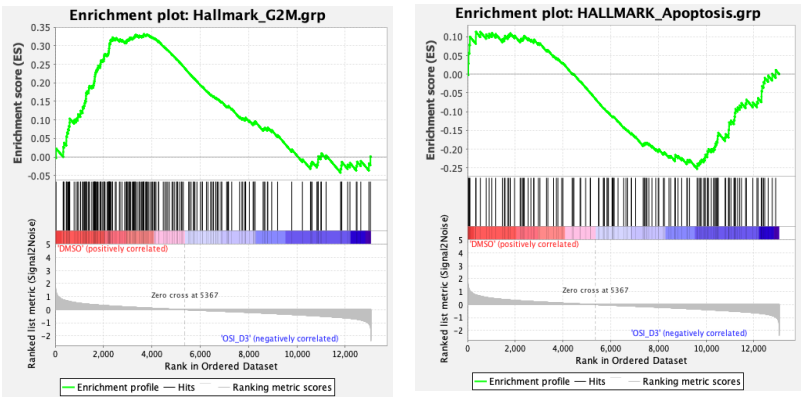
- Doubling rates controls should be included in the experimental protocol. Does the model double within the drugging experiment? The use of negative/positive controls is not clear.
Author response: As outlined above, we have now included quantitative data on organoid growth rates. We also highlight the importance of using clear-bottom 96-well plates to monitor growth rates and drug response during the experiment (page 4, line 153). The presented protocol has been used across several EGFR-mutant NSCLC organoid cultures, 4 of which are highlighted here across Figures 2 and 3. Overall, the protocol is optimized to work with the indicated times for EGFR-mutant NSCLC organoid cultures even if minor differences in growth

rate are observed. We have noted the necessity to compare the expected growth development over the initial 7-day period and adapt if needed (page 10, line 427-434). We hope the new quantitative data of organoid size and relative fold change in Figure 1B will provide the necessary detail for the readers to adapt culture times, should it be necessary (e.g., when working with organoids of other cancer or non-cancer entities).

We agree with the Reviewer that strong differences in doubling times can have an impact on drug responses, with recent work modelling the impact of differences in growth rate or cell density on response to cancer-targeted drugs (PMID: 27135972). We have expanded our discussion and emphasized the option to monitor organoid growth rate quantitatively (page 10, line 427-434). Please also see below for a detailed description regarding the observed phenotypes under drug treatment (cell cycle arrest and apoptosis).

Regarding necessary controls, we have provided data for sensitive and resistant organoids models (Figure 2) to show how different sensitivities to drug treatment are observed. We have provided context to benchmark sensitivity profiles by comparing to an established sensitive EGFR-mutant PC9 lung adenocarcinoma cell line model treated with osimertinib (Figure 3A). Additionally, every drug escalation treatment includes an untreated DMSO-control, in which no drug is added. This serves as an internal negative control in each experiment (no drug effect).

Upon targeted treatment, sensitive cells / organoids will undergo cell cycle G1 arrest and cease active proliferation. To demonstrate the described phenotype, we have analyzed changes in gene expression by gene set enrichment analysis (GSEA) for the G2M cell cycle gene set and the apoptosis gene set. As expression of G2M cell cycle genes is associated with active proliferation, a nominal enrichment of expression in the untreated control (DMSO) is expected. For apoptosis-related genes, a nominal enrichment in treated cells (OSI_D3) is expected. Both has been confirmed in the EGFR-mutant TH107 NSCLC organoid model treated with Osimertinib.



GSEA using bulk RNA sequencing data obtain for EGFR-mutant TH107 NSCLC organoids, comparing untreated control (DMSO) and cells treated with Osimertinib for 3 days (OSI_D3). GSEA for Hallmark G2M expression signature (left) shows enrichment in DMSO- treated cells. nominal enrichment score (NES): +1.708, FDR < 0.0001. GSEA for Hallmark Apoptosis expression signature (right) shows enrichment in Osimertinib-treated cells. NES: -1.075, FDR: ns, 0.3275.

- There is no indication that the viability of organoids is assessed before plating.

Author response: The viability of the organoid single cell suspension is ≥ 95%, as reported by the Vi-Cell XR Cell Analyzer before plating. This is done automatically by the cell analyzer in the counting process. Further, no significant number of dead cells is visible as monitored by brightfield microscopy at the day of seeding or the initial 7-day growth period. (Figure 1B) The need to monitor organoid growth and fitness is explained in the discussion, section "*Critical steps in the protocol*" (point iii). Further, we have added a NOTE at the counting step to indicate the need for viability assessment. (page 4, line 161-163)

- Details on the type of plates, tips, etc. should be included in the standardized protocol.
Author response: Culture plates for this experiment (96-well format) are listed in the Materials Table including commercial names, provider, and catalog number. In addition, we have now listed general items such as 15mL centrifuge tubes, pipettes, tips, 0.22 µM filter, etc. in the Materials Table, with provider information where it is critical.

- 7 days of growth prior to drugging is too long and it would depend on the organoid doubling time. Based on experience in the field, some models might overgrow at that point. More representative images are needed to assess the status of organoids after 7 days (must be based on multiple models tested with multiple drugs).
Author response: In Figure 1B, we have now added the comparison to the original culture with fully grown organoids as well as additional representative images for a second organoid model (TH330) and the quantification of changes in organoid size. We hope that this provides enough context for the readers to assess organoid development during the initial 7-day growth period. Of note, treatment initiation at an early organoid state is necessary as outlined by the Reviewer. As we have outlined above, a well-defined organoid size is observed at the day of treatment initiation. In brief, organoids show an ~ 2.4-fold change increase in size and an organoid diameter of approx. 30-40 µm at day 7 after seeding. Further, this time point is optimized to allow for a distinguishment of sensitive and resistant models as presented in Figure 2 and Supplementary Figure 1 [now Figure 3]. While we expect that the protocol is amendable to other oncogenic driver / inhibitor combinations and additional cancer entities, we hope to highlight profiling of drug sensitivities for NSCLC organoids more generally in the future. We have simplified our title to address this.

- Plating protocol lacks detail. It would be better to establish a number of cells that need to be plated per well as opposed to the number of cells needed to plate a full 96 well plate. Also, the number of cells used per well might not be the same for all the models and should be determined empirically based on the doubling rate of the model. Such a test should be a part of this protocol.
Author response: We have now indicated the total number of cells seeded per well in point 2.2.3, section a. We agree with the Reviewer that doubling time is an important aspect to consider when monitoring drug responses. We have optimized the presented protocol according to similar considerations. We now present quantitative data regarding changes in organoid size / doubling in Figure 1B to allow the readers to evaluate organoid growth during the experiment more accurately. We have extended our discussion to highlight this fact (page 10, line 427-434).

- Drugging protocol lacks detail. Is this based on manual drugging? Can it be extended to drugging with dispensers? If authors have extensive experience in this field they should be able to communicate both methods. Can this be extended to 384 well or 1536 well plates? 96 well plates are seldom used for the drugging of organoids; currently, the majority of papers that have reported the use of organoids for drug screening are using a 384 well format or a 136 well format.
Author response: The protocol is based on manual drugging using a multichannel or regular pipette. As outline in step 3, the protocol simply requires the aspiration of media and the addition of prepared drug solutions. Critical steps such as rotating the organoid plate to avoid aspiration of embedded organoids are indicated.

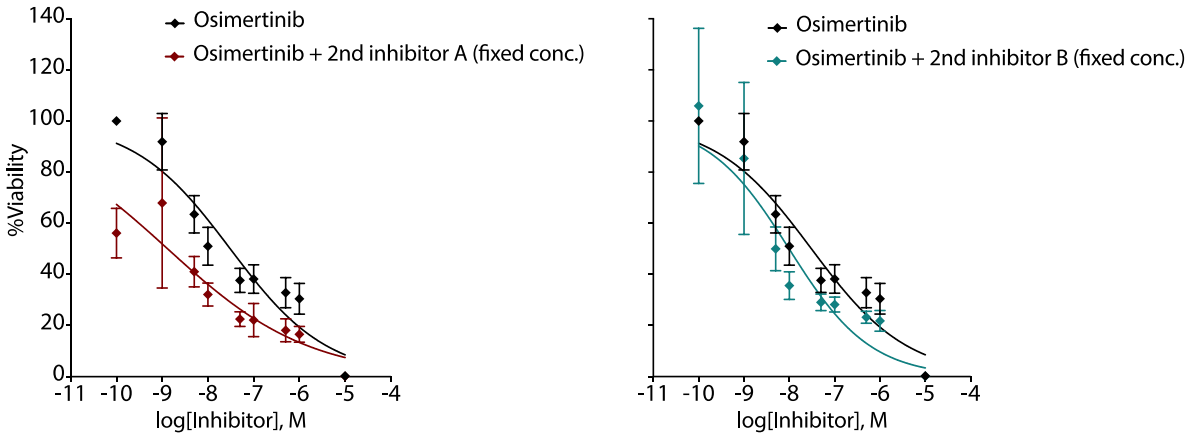
We have experience using media dispenser and liquid handling devices in cell line models. Given the limitations in organoids biomass and the high cost-factor for media production, we have not optimized the use of media dispensers with organoids. Similarly, we have not and

would not recommend the use of 384 and 1536 well plates at this point, given the variation when working with BME2 / matrigel – especially at smaller volumes than 5 µL.

- The only representative data shown is with few models and one drug (Osimertinib). More representative data is needed. More models should be tested with different drugs including chemotherapeutic agents. Is the same protocol used for chemo and molecular targeted drugs? Chemo drugs generally act faster than molecularly targeted drugs.
Author response: Our protocol presents the profiling of drug sensitivity in NSCLC organoids. NSCLC is a cancer entity with molecularly defined subsets harboring clear oncogenic drivers. Targeted therapies have significantly improved patient survival and safety during treatment. In EGFR mutant NSCLC, Osimertinib is a widely used and current FDA-approved EGFR inhibitor, that improves overall patient survival when used as first line treatment. We have included respective references in our manuscript (page 7, line 310-312). To highlight our aim regarding the profiling of NSCLC organoids and the focus on EGFR-mutant models, we have now adapted our title respectively. We believe that this protocol can be used to profile chemotherapeutic responses, with minimal alterations. However, we have not tested such agents given the reduced clinical relevance in our EGFR-mutant NSCLC setting.

- This protocol is not novel and is outdated. The majority of the papers that have published organoid establishment have used similar or more advanced protocols with far more detail.
Author response: We apologize for the lack of clarity regarding our main focus. Our aim is to profile drug sensitivity in NSCLC cancer organoids, while we rely on and reference establishment protocols. We have now included a brief paragraph at the beginning of our manuscript regarding organoid establishment, linking to our collaboration partners and previous literature on organoid establishment. We have also updated our title to highlight our distinct aim of profiling drug sensitivities rather than organoid establishment.

- Authors refer to using this protocol for combination treatment yet there are no step-by-step instructions on how to set up combination treatment plates or how to analyze them. Furthermore, no representative data is shown for combination treatment.
Author response: We are discussing the potential use of the protocol for combination treatments as an adaptation. In general, we are using either i) a combination of one fixed dose for the additive drugs while escalating our main inhibitor or ii) complex matrix-based drug combinations escalating both drugs against each other to allow the calculation of Loewe, Bliss or HSA combination or synergy scores in cell line systems. Both options could be implemented using our organoid protocol and only the preparation of drug dilutions would have to be adapted in a way that it addresses the experimental question. For organoids, we prefer to combine a fixed additive drug while escalating the main inhibitor, allowing us to include more replicates and accounting for the higher standard deviation observed in organoid sensitivity profiling. Please find a representative example below. Please note that we are not able to share this data at this point as it is included in another publication in preparation.



Combinatorial drug treatment in EGFR-mutant TH107 organoid model treated with Osimertinib escalation in presence of a second additive inhibitor at fixed concentration.

We have now indicated this example for combinatorial treatment approaches in the discussion (page 11, line 509-512). We have adapted keywords to not overemphasize combinatorial treatments. We have not made further adaptations to the protocol in this regard as only the used drug dilutions that organoids are treated with would differ to allow for the testing of combinatorial treatments.

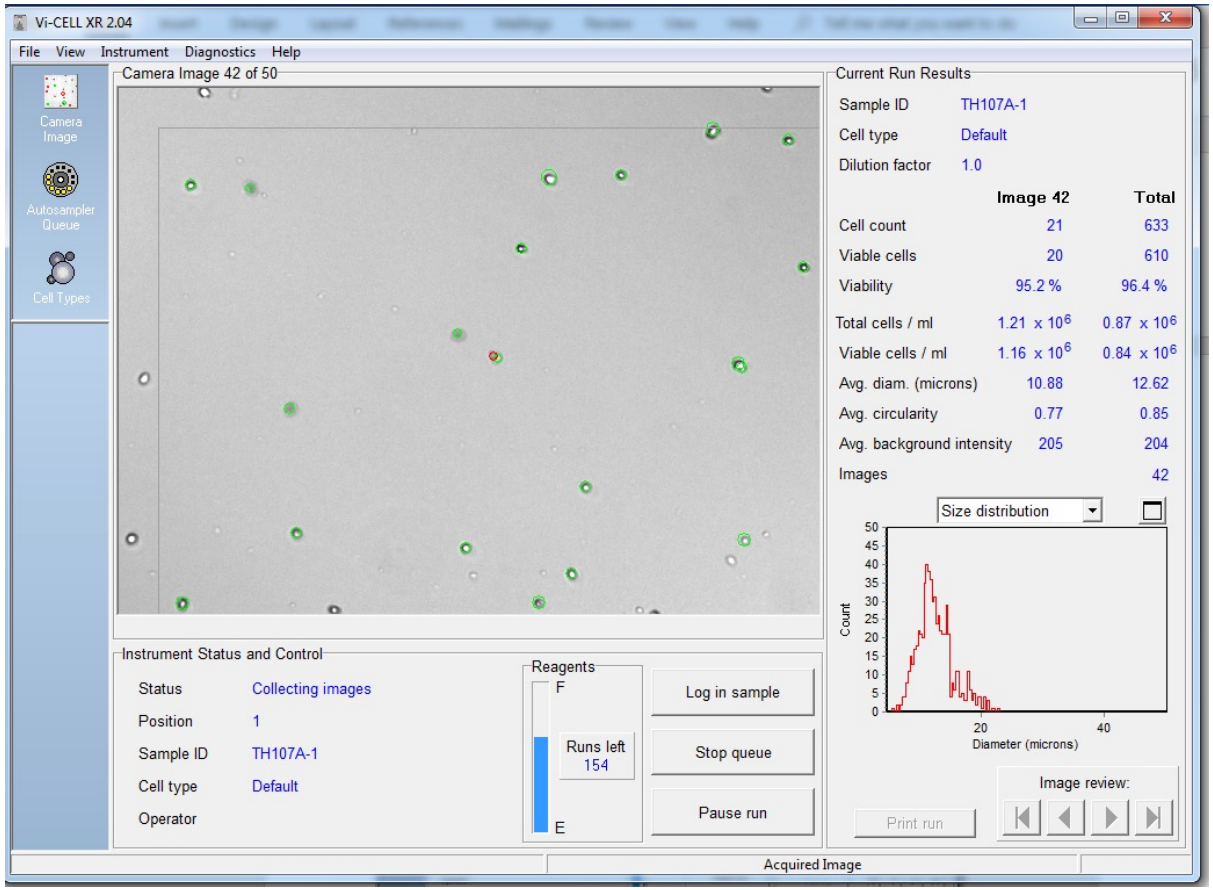
Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Suppl-Fig1-R2.pdf

Vi-CELL XR 2.04

Beckman Coulter, Inc.

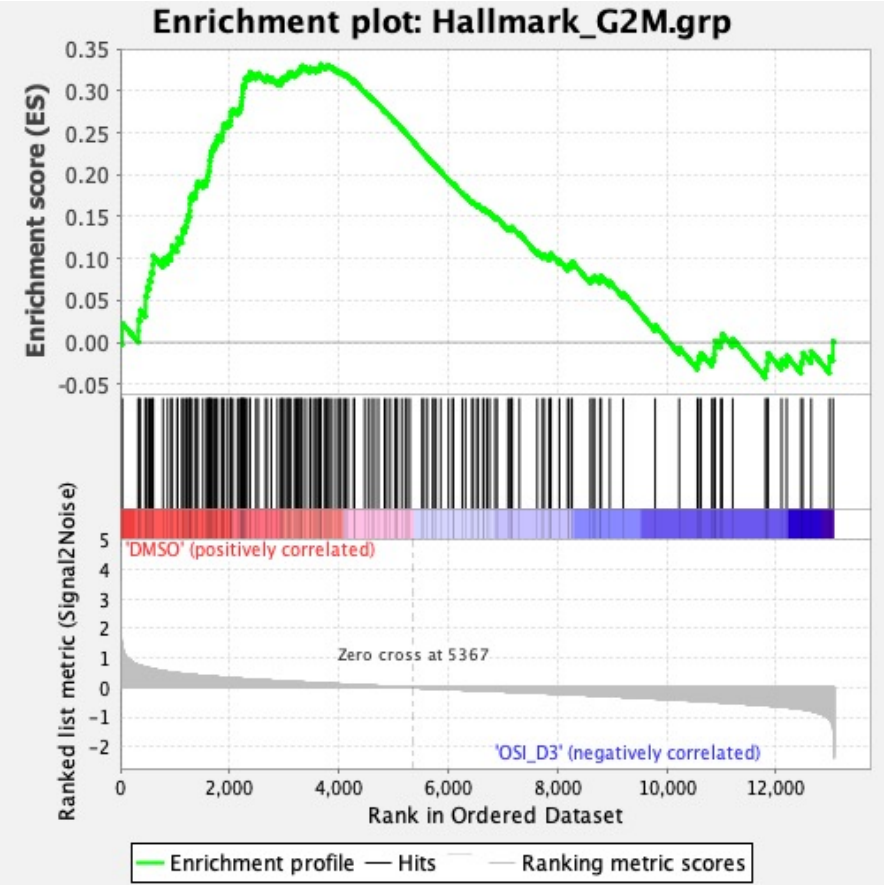
Sample ID	Viability (%)	Total cells /ml (x10^6)	Viable cells /ml (x10^6)
TH107A-1	96.4	0.87	0.84
TH107A-2	96.5	0.92	0.88
TH107A-3	96.6	0.82	0.80
TH107B-1	96.2	0.85	0.82
TH107B-2	97.2	0.86	0.83
TH107B-3	95.8	0.80	0.77
TH107BC-A-1	94.6	1.08	1.02
TH107BC-A-2	94.3	1.05	0.99
TH107BC-A-3	96.1	1.00	0.96
TH107BC-B-1	95.8	1.13	1.09
TH107BC-B-2	95.8	1.15	1.10
TH107BC-B-3	94.8	1.10	1.04



Supplemental Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc);Suppl-Fig2-R2.pdf

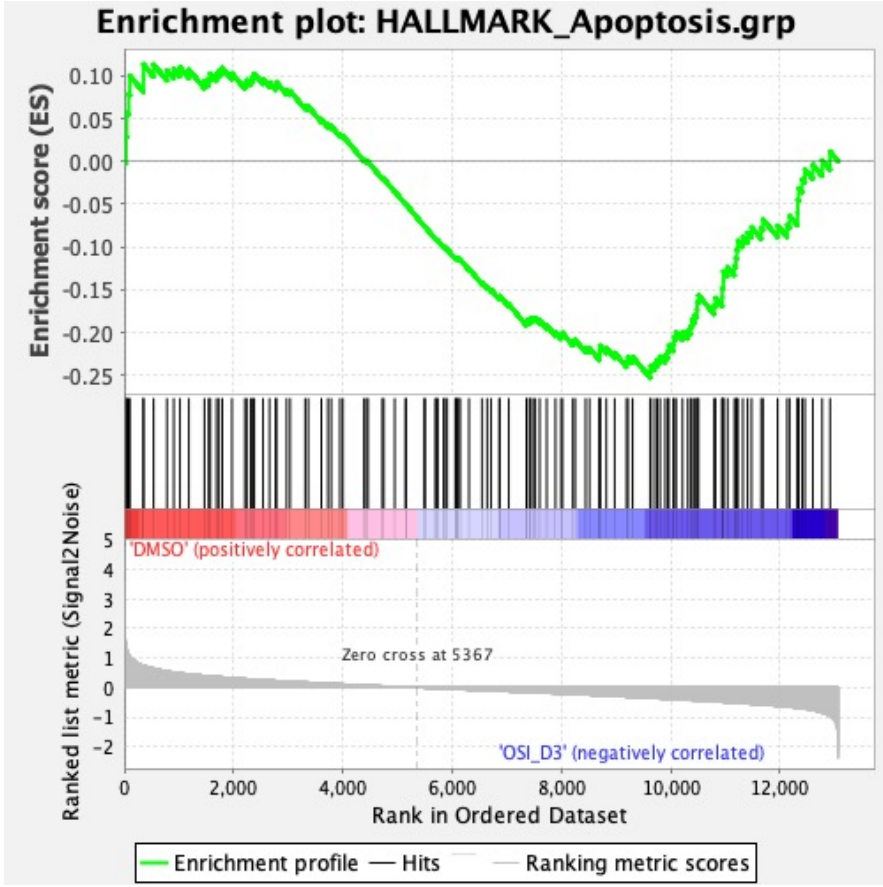
A



DMSO

Osimertinib 1 μM (day 3)

B

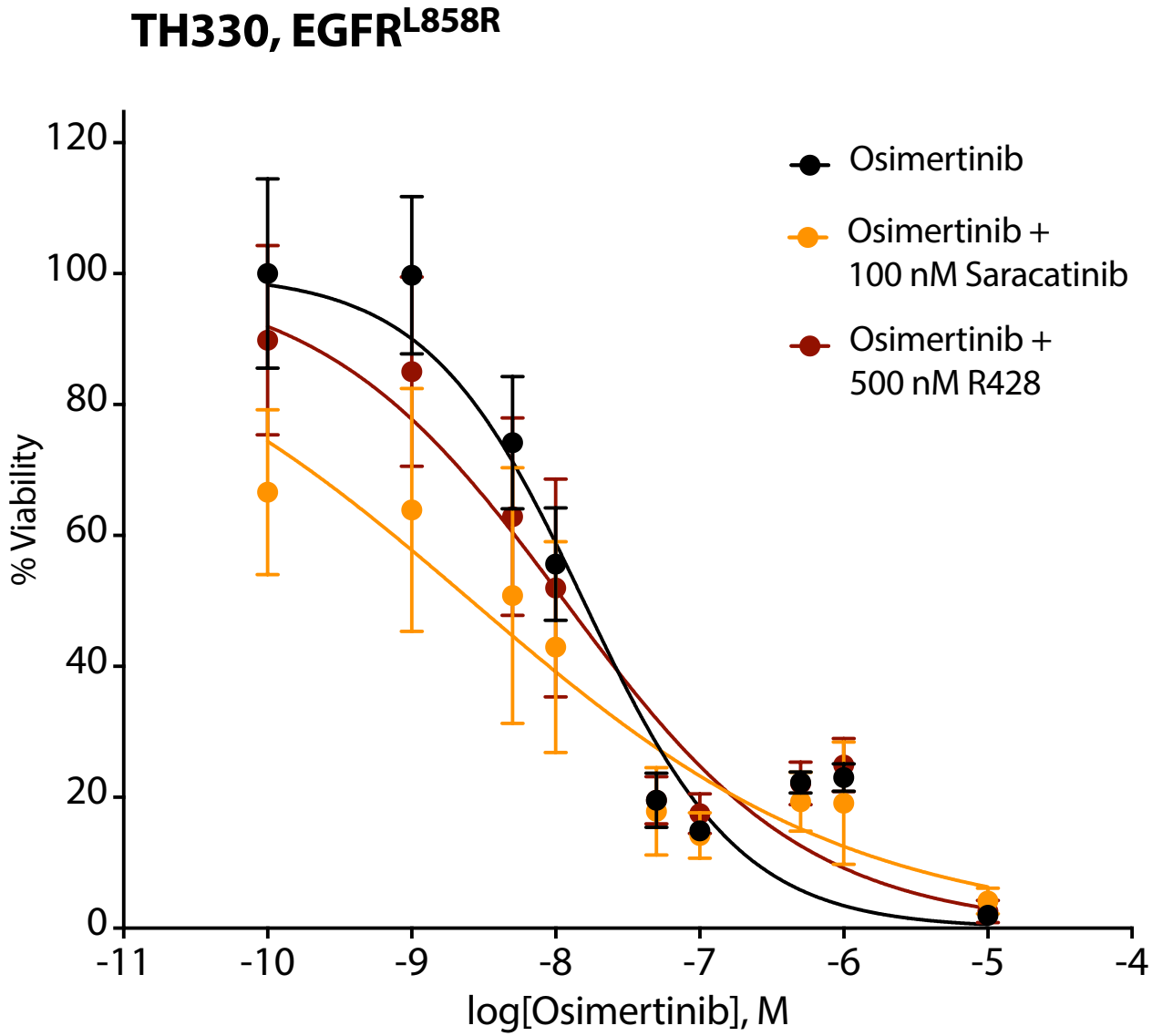


DMSO

Osimertinib 1 μM (day 3)

Supplemental Figure 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Suppl-Fig3-R2.pdf



Supplementary Table 1

A	Transpose of Organoid-growth_TH107		
	average size [µm]	SD	N
d0	11.00	2.00	41
d4	15.89	5.40	90
d7	26.13	8.32	73
	Rel fold change		
d0	1.00		
d4	1.45		
d7	2.38		

B
d0
d4
d7
d0
d4
d7

Transpose of Organoid-growth_TH330		
average size [µm]	SD	N
15.32	2.81	90
23.26	5.71	134
36.96	10.26	172

Rel fold change
1.00
1.52
2.41