High-throughput spheroid screens using Volume, Resazurin reduction and Acid phosphatase activity

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Summary

Mainstream adoption of physiologically-relevant three-dimensional models has been slow in the last 50 years due to long, manual protocols with poor reproducibility, high price and closed commercial platforms. This chapter describes high-throughput, low-cost, open methods for spheroid viability assessment which use readily-available reagents and open-source software to analyse spheroid volume, metabolism and enzymatic activity. We provide two ImageJ macros for automated spheroid size determination - for both single images and for images in stacks. We also share an Excel template spreadsheet allowing users to rapidly process spheroid size data, analyse plate uniformity (such as edge effects and systematic seeding errors), detect outliers and calculate dose-response. The methods would be useful to researchers in preclinical and translational research planning to move away from simplistic monolayer studies and explore 3D spheroid screens for drug safety and efficacy without substantial investment in money or time.

Key words

alamar blue, viability assays, overlay culture, hanging drop, FiJi ImageJ, image analysis, three-dimensional cell culture, in vitro model, preclinical screening, drug sensitivity

1. Introduction

The purpose of this chapter is to describe the detailed practical procedures behind three complementary techniques (volume, resazurin reduction and acid phosphatase activity) for spheroid viability assessment in high-throughput 96-well format *(1)*. Spheroids are self-organised three-

dimensional (3D) aggregates of cells displaying physiologically relevant gradients of oxygen, nutrients, cell-cell and cell matrix interactions *(2, 3)*. Aggregate cultures were first described in the 1950s by Moscona *(4)*, and the advantages of using spheroids in cancer research were recognised in the 1970s by Sutherland *(5)*. However, poor reproducibility, large variation in spheroid size, lengthy hand-operated manipulations and low-throughput precluded the introduction of spheroids in mainstream drug screens. The introduction of plate-based platforms for spheroid culture in hanging-drop *(6, 7)* or liquid overlay *(8, 9)* has enabled researchers to produce a single-spheroid per well and control spheroid size in a high-throughput format. This has stimulated the adoption of spheroid screens for drugs targeting dormant tumour cells *(10)*, spheroid assays modelling chemo and radio-resistance *(11, 12)* strategies for sensitising hypoxic cells in the tumour core *(13, 14)* and drug safety assays *(15, 16)*.

We present a suite of three multiplexable methods to assess spheroid health in overlay spheroid cultures. The plate format used is compatible with standard plate-readers, and the methods rely on generic reagents available at lower costs compared to ready-made kits. The techniques use open-source software for image analysis (Fiji distribution of ImageJ *(17, 18)*) and do not require investments in new equipment or software. The practical application of these methods and the characterisation of linearity and sensitivity have been discussed in our recent publication *(1)*. These methods are suitable for monocultures (one cell-type) and co-cultures (mixed cell-types). In co-cultures, the volume or metabolic activity of the co-culture spheroid can be used as a proxy measure for the total number of cells, subsequently the proportion of each cell type can be quantified with microscopy or flow cytometry *(19)*.

Manual spheroid size measurements and morphological characterisation of 3D aggregates have been extensively used from the earliest days of spheroid research *(4, 5, 20)*. With the development of overlay and hanging-drop plates, many proprietary platforms (Celigo, Zeiss, Perkin-Elmer etc)

have been employed in measuring spheroid size and estimating volume in high-throughput *(8, 21)*. These platforms require substantial investment in equipment and image analysis software, which may not be available to all research labs and may slow down the adoption of spheroid screens. We have written two macros which automate spheroid size analysis on the bioimaging Fiji *(17)* distribution of the open-source image analysis platform ImageJ *(18)*. The first macro works on manually acquired images from simple setups of camera-equipped brightfield microscopes, where spheroid images are recorded as separate files in a folder. It is compatible with computers with less than 2GB of RAM which may struggle to load all images in a single image sequence. The second macro is substantially faster and works with image sequences (stacks), for example images taken with automated-stage microscopes, which often produce stacks of multiple images in one file. It can also be used on separate images imported as an image sequence in ImageJ (**see Note 10**). Estimating spheroid viability solely based on spheroid size can be misleading because:

(1) Cell densities can be different in the different regions of the spheroid (eg hypoxic or necrotic core)

(2) Cells can shrink without dying in response to drug treatment

(3) Dead cells can form a large proportion of the spheroid at drug concentrations above the IC50

Methods to alleviate these shortcomings use, for example, dyes which detect dead cells (propidium iodide, 7-aminoactinomycin-D, ethidium homodimer) or employ immunohistochemistry assays on fixed spheroids with markers of apoptosis (e.g. Caspase-3). However, there are currently no high-throughput methods to probe spheroid histology and protein expression. Another approach would be to use metabolism-based assays for spheroid health assessment.

The resazurin (Alamar Blue) assay measures spheroid metabolism of live cells, which reduce resazurin to the fluorescent product resorufin *(22)*. If purchased as resazurin powder and prepared

in-house, the assay is thousands of times cheaper than the ATP-based kits. The resazurin assay does not kill or lyse the spheroids and the latter can be harvested for dissociation and cell counts, flow cytometry or histology 4 h after Resazurin addition*(1, 19)*. Its main limitation compared to the ATP-assay is that both resazurin and the fluorescent resorufin need to distribute throughout the spheroid and back into the medium to be quantified. For spheroids where the cells form tight-junctions and impede dye diffusion, treatment with chelators (Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid- EGTA) has been suggested to improve the reliability of the assay *(23)*. Regardless of dye diffusion, all metabolism-based assays (ATP included) would detect less metabolically active cells (e.g. in the hypoxic core) as "less-alive" and can potentially underestimate dormant-cell viability. Furthermore, Chan et al have demonstrated that certain anticancer compounds elicit a spike in metabolic activity at cytotoxic concentrations resulting in overestimation of viability *(24)*.

The third assay described is the acid-phosphatase assay advocated by Friedrich *(21, 25)*. It measures acid phosphatase activity as a proxy for cell viability and involves lysing the cells, thus eliminating the problems with diffusion and substrate penetration. While excellent results have been reported with tumour cell lines with this assay *(21, 25)*, it overestimated spheroid viability in etoposide-treated primary cultures of human fetal neurospheres *(1)*.

We have used the three viability assays in a variety of human medulloblastoma cells lines *(26)*, patient-derived early passage medulloblastoma cultures and human fetal brain-derived neurospheres *(1, 19)*. The linearity of assay signal in the physiologically-relevant size-range for each cell line has been determined by plotting assay readout against the number of cells per spheroid. The protocols below give an in-depth view of the practical requirements for optimal assay performance.

2. Materials

2.1 General cell culture reagents

1. DMEM (Dulbecco's Modified Eagle's medium): 4.5 g/L high-glucose with 1 mM sodium pyruvate.

2. Ham's F12 medium with 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).

3. 200 mM L-Glutamine solution.

4. Accutase cell-dissociation solution. Aliquot and store at -20°C. Thaw in the fridge before use.

5. Fetal bovine serum (FBS).

6. Full cell culture medium. For 100mL: 44.5 mL DMEM, 44.5 mL Ham's F12, 1 mL L-Glutamine solution, 10 mL fetal bovine serum.

7. Phosphate buffered saline (Ca^{2+} and Mg^{2+} free), sterile solution (PBS).

8. Cell culture treated flasks.

9. Ultra-low attachment round bottom 96-well plates (Corning, 7007).

10. Plastic paraffin film

2.2 Fetal brain culture

1. B27 supplement without insulin (50X).

2. N2 supplement (100X).

3. Heparin sodium salt from porcine intestinal mucosa (Sigma Aldrich, Grade I-A, cell culture tested). Prepare a 5 mg/mL stock solution in sterile-filtered deionised water and store at 2-8°C for up to 6 months.

4. Human serum albumin: lyophilised powder

5. Recombinant human epidermal growth factor (EGF). Prepare a 20 µg/mL stock solution in sterile-filtered 0.3% wt/vol (human serum albumin in PBS), aliquot and store at -20°C for up to 1 year.

6. Recombinant human basic-Fibroblast growth factor (FGF). Prepare a 10 µg/mL stock solution in sterile-filtered 0.3% wt/vol (human serum albumin in PBS), aliquot and store at -20°C for up to 1 year.

7. Neural stem cell medium. For 100mL: Filter 47.5 mL DMEM and 47.5 mL F12 through a 0.22 µm filter and then add 2 mL B27, 1 mL N2, 100 µL Heparin , 100 µL EGF stock (20 µg/mL), 100 µL FGF stock (10 µg/mL) (**See Note 1**).

8. Non-treated cell culture suspension flasks with filter cap, 25cm^2.

9. Polystyrene conical tubes 15ml. **(See Note 2).**

2.3 Resazurin

1. Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+} (HBSS).

2. Resazurin (high purity biological stain quality). Resazurin stock solution: dissolve 12 mg resazurin in 100 mL Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+} (HBSS). Filter through 0.22µm filter to sterilise. Aliquot and store protected from light at -20°C for up to a year. Thaw just before use and store any leftover stock solution at 2-8°C for no more than 2 weeks. Avoid repeated freeze-thawing.

2.4 Acid phosphatase

1. Triton-X 100 solution.

2. Para-nitrophenyl phosphate (PNPP, N4645, Bioreagent, Sigma-Aldrich). Store at -20°C.

3. Sodium acetate 3M solution: Dissolve 20.41 g Sodium acetate trihydrate in 40 mL deionised water. Adjust pH to 5.2 with glacial acetic acid in the fume hood. Adjust volume to 50mL and filter-sterilise (0.22 µm filter).

4. Sodium hydroxide 1M: Make 4g NaOH up to 100 mL with deionised water.

5. Acid phosphatase buffer (APH buffer): 0.1M Sodium acetate and 0.1% Triton X (45ml). Prepare 40mL and store at 2-8°C for up to 4 weeks. Add 1.5 mL of 3M sodium acetate buffer to 45 µL Triton X. Make up to 45 mL with sterile deionised water.

6. APH assay solution. Prepare immediately before the assay. Dissolve 18 mg of 4-Nitrophenyl phosphate in 9 mL APH buffer. Used the solution immediately. Do not store and protect from light.

3. Methods

3.1 Propagation of fetal brain tissue as neurospheres

1. Seed cells 1-2 x 10^6 cells in 5 mL NSC medium in a non-treated 25cm^2 flask. (**See Note 3**).

2. Feed the neurospheres by adding 2mL of fresh medium every 2-3 days and change the medium and the flask if the neurospheres start attaching to the surface of the flask. (**See Note 4**).

3. Dissociate the neurospheres every 7 days or when they reach a diameter around 100-300 μm. (**See Note 5**).

3.2 **Dissociation of neurospheres**

1. Remove neurosphere cell suspension from culture flask and transfer to a 15 mL polystyrene conical tube and centrifuge (50 g, 30 s).

2. Aspirate medium leaving a maximum of 100 μL of medium remaining.

3. Resuspend neurospheres in 2-5 mL PBS and centrifuge (50g, 30 s).

4. Aspirate the PBS leaving a maximum of 100 μL of PBS remaining.

5. Resuspend the neurospheres in 1 mL Accutase and shake in a water bath (37°C, 5 min).

6. Pre-wet a 1 mL pipette tip and pipette the cloudy neurosphere suspension up and down to get a single cell suspension.

7. Add 2mL medium to stop the dissociation reaction and centrifuge (300 g, 5 min)

8. Gently aspirate the supernatant and resuspend cells in 200 μL PBS, using the 200μL pipette, then add 800μL fresh medium and perform a cell count.

3.3 Propagation of tumour cell lines

Culture as required by the cell line. For UW 228-3 medulloblastoma cells:

1. Seed 250,000 - 500,000 cells in a 75cm^2 cell culture treated flask, add fresh medium every other day and completely change the medium every 4 days. Subculture to 75-85% confluency.

2. Dissociate by removing the medium, washing with PBS, incubating with 4 mL Trypsin (5 min), stopping the reaction with 6 mL fresh medium, centrifuging (300g, 5 min) and resuspending in 1 mL PBS. Add fresh 1-4 mL medium and perform a cell count.

3.4 Spheroid formation in ultra-low attachment plates (day 0)

1. Prepare a single cell suspension from the cell line/primary cells to be used and perform a cell count.

2. Pipette 200 µL/well PBS in all wells of lines A and H (top and bottom) of the 96-well plate. (**see Note 6**).

3. Add 100 µL/well medium in column 1 (wells B to G).

4. Seed cells in 100 µL/well medium in columns 2 to 11 (wells B to G) (See **Note 7**)

5. Wrap each plate in plastic paraffin film along it's perimeter to securely hold the lid and bottom together.

6. Centrifuge in a plate centrifuge (100g, 3min), remove plastic paraffin film and place in the incubator.

3.5 Optimising spheroid seeding density

1. Prepare serial dilutions of the cell suspensions with cell counts ranging from 5000 to 200,000 cells per ml.

2. Seed 100 µL from the different dilutions as six technical replicates per plate column.

3. Centrifuge plate (100g, 3min) and place in incubator.

4. Monitor spheroid volume and perform resazurin and APH assay at the appropriate time point. (See the acceptance criteria section for picking spheroid size). (See **Note 8)**

3.6 Spheroid drug sensitivity screens

1. Prepare a single cell suspension of the primary cells or cell line of interest and dilute to the predetermined seeding concentration.

2. Seed 100 µL/well medium without cells in column 1 and 100 µL/well of the cell suspension in columns 2-12.

3. Centrifuge plate (100g, 3min) and place in incubator for the pre-set spheroid growth period (3-4 days).

4. Image plates on day 1 and every other day if needed. Image just before drug addition.

5. On day 3 or 4, prepare a 2x serial dilution series of the drug to be analysed in medium. Use nine half-logarithmically spaced concentrations (0.3, 1, 3 µM etc.) centred along the estimated IC50 or the potency values reported in the literature. (See **Note 9).**

6. Add 100µL to the appropriate wells as shown in **Table 1** :

7. Incubate for 48-72h, exchange medium (refresh drug solutions every 48-72h, depending on drug stability in medium).

8. Proceed to volume, resazurin and APH analysis.

3.7 Spheroid volume analysis

1. Image spheroids from day 1 onwards (24h after seeding) when required.

2. Collect phase-contrast brightfield images either manually or with a microscope equipped with an automated programmable stage (preferred).

3. Download the Fiji distribution of ImageJ (http://fiji.sc/) and start the programme.

3.7.1 **Image analysis steps for macro ver1 (slower)**: use for manually collected images (collection of spheroid images in a folder)

1. Load macro by dragging and dropping the **Macro1.ijm** file onto the main ImageJ window. Alternatively open the Macro editor (*Plugins/New/Macro*) and paste the **macro1** text, select "*Language/ImageJ1 macro*".

2. Measure the scale of your image (number of pixels equal to 100 μm) and enter the value after "*distance=*" in line 6 in macro1.

3. Start the macro by pressing the *Run* button in the Macro editor window. There should be no images open when starting the macro.

4. Select an input folder containing the original spheroid images. The input folder should only contain images and no other files.

5. Select an output folder which is going to contain the quality control images with spheroid outlines.

6. Wait for the macro to finish, progress is displayed in the status bar of the Main window

7. Analyse the results as described below.

3.7.2 **Image analysis steps for macro ver2 (faster):** use for automatically acquired images in an image sequence (collection of images in a single image file- image stack or hyperstack)

1. Load macro by dragging and dropping the **Macro2.ijm** file onto the main ImageJ window. Alternatively open the Macro editor (Plugins/New/Macro) and paste the **macro2** text, select "*Language/ImageJ1 macro*".

2. Load image stack (sequence) containing spheroid images. Drag the image file onto ImageJ's main window or open using "*File/Import/BioFormats*". In the BioFormats Import options use the "*Hyperstack*" option and tick "*Open all series*", "*Concatenate series…*" and "*Autoscale*". A spheroid image sequence appears with a slider at the bottom to view all spheroid images. (**See Notes 10 and 11**)

3. If the image displays size in pixels, rather than metric units, apply the correct predetermined scale. ("*Analyze/Set Scale*").

4. Start the macro by pressing the *Run* button in the Macro editor window.(**See Note 12**)

5. A window will appear showing an overlay of all spheroids. Use the rectangular selection tool to select the smallest area of the image containing all spheroids and press "*OK*" in the "*Action Required*" window. (**See Note 13)**

6. A second window of the spheroid image sequence will appear and another "Action required" pop-up window will prompt you to select a threshold. Go to *Image/Adjust/Threshold* (shortcut: Select the image window and press Shift+T). (For manual thresholding continue to step 7, for the code to automate the next two steps **See Note 14)**

7. Use the sliders to select a threshold which identifies all spheroids (red) and selects little background. (**See Note 15**)

8. Press Apply in the "*Select Threshold window*", then answer "*Yes*" in the window asking "*Process all images?*". Some Fiji versions (e.g. 64-bit) display a "Convert stack to binary" window, where you just need to click "OK". Afterwards press "*OK*" in the "*Action required*" window. **(See Note 16)**

9. Use the ROI manager to cycle through all outlined spheroids in the stack, check for failed image recognition and remove false positive spheroid outlines **(See Note 17**). The macro will produce two new files in the same folder as the original image sequence. A "Filename Quality control (QC)" file

containing the spheroids along with their recognised outlines and a "Filename Results" file

containing all measurements.

3.7.3 Analysing the spheroid measurements from ImageJ in Excel (See Note 18)

To streamline data analysis we have developed an Excel template which calculates the radius and

volume of an equivalent sphere from spheroid area. The template is optimised for plate designs

incorporating 6 consecutive technical replicates, first condition untreated control and up to 10

treatment conditions.

Measurement values from the ImageJ analysis need to be pasted in cells B1 to F67. The panels in

blue are calculated values based on the measured spheroid area. (**See Note 19**)

1. Open the "Filename Results.csv" file., select columns "FeretX", "FeretY" and "FeretAngle" and delete

   them leaving only the column with image numbers, and the ones labelled "Label", "Area", "Feret"

   and "MinFeret" in columns "A" to "E" in Excel.

2. Click on any cell containing data and select all cells with data (Ctrl+A) and copy (Ctrl+C)

3. Open the "Volume analysis.xlsx" template file

4. Paste the copied values from the Results file into the template by selecting cell B1 and pasting from

   the clipboard (Ctrl+V). **(See Note 20)**

5. Remove outliers and paste data in GraphPad Prism.

3.8 Spheroid metabolism analysis (Resazurin assay)

1. Thaw the resazurin stock solution

2. Prepare a working resazurin solution (12 mL per plate) by adding 1.6 mL Resazurin stock to 10.4 mL

   of fresh medium. Wrap in aluminium foil to protect from light.

3. Remove spent medium from the plate wells to leave 50 μL in each well.

4. Add 150 μL from the working resazurin solution to all wells.

5. Leave the plate in the incubator (37°C, 4 hours).

6. Read fluorescence with a plate reader at 530-570 nm excitation and 580-600nm emission. **(See Note 21)**

3.9 Acid phosphatase assay

1. Remove old medium from the plate wells leaving a maximum of 50 µL and replace with 150µl PBS.

2. Repeat step 1.

3. Remove 100µl of PBS from each well, leaving 100 µL of PBS.

4. Add 100 µL fresh APH assay solution.

5. Leave in the incubator (37°C, 90 min).

6. Add 10µL 1M NaOH solution to each well and record absorbance (405 nm) within 10 min.

3.10 Dissociating spheroids to determine number of cells per spheroid.

1. Collect 6-12 technical replicate spheroids per condition in 1.5 mL centrifuge tubes.

2. Leave the spheroids to settle to the bottom of the tube (1-2 min) or centrifuge (100g, 30s).

3. Replace culture medium with PBS and centrifuge (100g, 30sec).

4. Add 200 µL Accutase and incubate at 37°C (5-20 min) with shaking.

5. Using a 200µl pipette take up and dispense the cloudy suspension up and down to get a single cell suspension.

6. Stop the dissociation reaction by adding 800µl fresh medium and centrifuge (200g, 5 min).

7. Remove the supernatant and resuspend in 50-100 µL of suitable Ca and Mg^{2+}-free buffer (PBS) to perform a cell count.

3.11 Data analysis in GraphPad Prism (versions 6 and 7)

1. Open GraphPad prism and select an X-Y Graph with 6 replicates for Y.

2. Write "[Drug name]" for "X-title" and paste the decimal logarithms of the concentrations from the Excel template file cells M16 to M26 in "X".

3. Name Group A with the experiment or cell line name and paste the individual well readings for volume, resazurin or acid phosphatase in "Y1 to Y6".

4. Normalise data by clicking *"Analyze/Normalise"* and define 100% as untreated control and 0% as positive control (25 %DMSO treated wells, 0 for Volume measurements*).*

5. Fit a sigmoidal dose-response curve:

"Analyze/Nonlinear Regression/Sigmoidal, 4P, X is log (concentration)". Fitting method: *least squares*, Constrain top to 100, constrain bottom >0, adjust range to fit points only up to the highest concentration of the drug used.

6. Inspect curve and reject if inappropriate or goodness of fit value "R square" is lower than 0.7.

3.12 Acceptance criteria

1. The readouts from the APH and resazurin assay would only be useful if there is good separation between the signal in wells containing spheroids from the background of medium-only wells or wells with dead spheroids (positive cell death control, 25% DMSO). Use the readout values for the untreated (medium-only or drug-carrier) and the positive cell-death controls (25%DMSO) to calculate the Z-factor *(27)*:

$$Z = 1 - \frac{3*(SD_{untreated\_control} + SD_{positive\_control})}{Mean_{untreated\_control} - Mean_{positive\_control}}.$$

Acceptable separation between the two readouts is considered when Z > 0.4. For spheroid size optimisation select spheroid sizes which consistently give signals with Z-factors above 0.4 compared to cell-free medium controls.

2. For volume, resazurin and APH- accept readouts with Coefficient of variation <20% after outlier elimination (empty wells).

4. Notes

1. Both EGF and FGF are unstable in cell medium and the latter should be used within a week or two after making.

2. Fetal brain cells can adhere to polypropylene plastics. Use only polystyrene tubes when handling these cells.

3. Always pre-wet tips and pipettes before aspirating the spheres to prevent them from sticking to the plastic.

4. Healthy cultures without extensive cell death do not require complete medium exchange. If there are many single cells 72h post seeding or extensive amounts of debris it is recommended to change the medium completely by gently spinning the small neurospheres in a 15ml polystyrene conical tube (100 g, 3-4 min). Often some of the spheres will try to attach to the surface of the flask but they can be dislodged by gently tapping the flask every 1-2 days or through adding fresh medium and gently washing the adherent spheres. In cases of extensive attachment change both the medium and the flask.

5. If there is a large discrepancy in sphere sizes you can separate the small (d<200 μm) spheres from the large (d>500 μm) by placing the spheroid suspension in a 15 ml polystyrene conical tube leaving the spheres to settle for 1 min. The supernatant will contain small spheres and the large ones will settle to the bottom. The large spheres can be dissociated and the small ones can be cultured until they reach d≈300μm.

6. The outer wells of the plate can suffer from increased evaporation, affecting medium osmolarity and cell viability. That is why it is recommended to fill them with PBS (lines A and H), use them as cell free medium controls (column 1, wells B to G) or complete cell kill controls (column 12, wells B to G).

7. We use six technical replicates per plate as often spheroids can be lost in medium exchanges.

8. Most often spheroid size of 300-500 μm is chosen based on the diffusion distance of oxygen and the formation of nutrient gradients. However the formation of hypoxic and nutrient deprived regions would depend on the cell type, the extracellular matrix produced, the presence of tight-junctions and the nature of the cell-cell interactions. These differ for each cell line and hypoxia can be ascertained by staining for markers (Hypoxia inducible factor-1α, Pimonidazole, Carbonic anhydrase-IX).

9. Aim for at least two drug concentrations with little effect on cell viability (top for cytotoxic drugs) and at least two with the maximum effect (bottom) to get a good curve fit in the non-linear

regression analysis. Make sure that the concentration of drug solvent is the same in every well and kept below 0.5% for DMSO. Also note that DMSO can deactivate some drugs such as the platinum complexes *(28)*.

10. This macro can also work on separate images in a folder by importing them as an image sequence by selecting *"File/Import/Image Sequence"*.

11. The macro tries to analyse the most recently opened file and that is why users should open the macro first and then the image.

12. The only open image should be the sequence you want to analyse.

13. The macro will produce a minimum-intensity projection of all images in the stack to help you identify the smallest area of the image which contains all spheroids. A pop-up window will appear prompting you to select an area to crop.

14. Thresholding has been left to manual selection in this macro but it can be automated if the same pixel intensity values recognise the spheroids in a series of image sequences. This further speeds up the process and allows the user to skip steps 7 and 8. To use automatic thresholding replace lines 28-31 of the macro with the following code:

    //This is an example of automatic thresholding using the "Yen" algorithm and selecting all pixels with intensity values from 234 to 1222 as spheroid on a light background

    run("Threshold...");

    setAutoThreshold("Yen");

    setThreshold(234, 1222);

    setOption("BlackBackground", false);

    run("Convert to Mask", "stack");

    //end of automated thresholding code

15. Untick "*Dark background*", try different thresholding algorithms to see which one does the best job in recognising spheroids from background in your particular imaging set. The macro can be tweaked to recognise spheroids within a certain size limit in line 39:

run("Analyze Particles...", "**size=50000-Infinity** show=[Overlay Outlines] display summarize add in_situ stack");

The default setting would recognise all spheroids with area larger than 50,000 μm^2 (d≈250 μm). The user can change those values to suit their spheroid size and eliminate small specks of false-positive dark background or to include spheroids smaller than 250 μm.

16. The "*Process all images?*" window can appear hidden behind the "*Action required*" window. Always press "*Yes*" in the former before clicking "*OK*" in the latter. Do not close the Threshold window manually, the macro will close it automatically after you press OK in the "*Action required*" window.

17. You can remove incorrectly recognised spheroids by selecting and deleting their outlines in the ROI manager. Missed spheroids can be outlined with the selection tools and added to the ROI manager (keyboard shortcut T). Update spheroid outlines on the image by selecting "Image/Overlay/Remove overlay" and "Image/Overlay/From ROI manager". If you make any deletions or additions to the outlines in the ROI manager, re-measure the spheroids by selecting all ROIs. In the ROI manager window click on one of the serial numbers and press Ctrl+A, then press the Measure button. Save the results file, the first number in the label corresponds to slide number.

18. The macros produce image files in the output folder (macro1) or a single quality control "Filename QC.tiff "image in the parent directory (macro2). They also save a "Filename Results" file, which can be opened in Excel. The most useful measurement outputs are spheroid area, Feret (maximum) diameter and MinFeret (minimum) diameter. Spheroid area is a more robust (less variable) measure of spheroid size compared to the maximum and minimum diameters and we have used it throughout our experiments to calculate spheroid diameter and the volume of an equivalent sphere.

19. The top right panel (N2 to X7) reconstructs the plate arrangement and color-codes spheroid volume values compared to the median spheroid volume for the whole plate. White cells encode empty wells, green cells are used for spheroids with 30% larger volume than the median plate volume, and red cells are for spheroids smaller than 70% of the median spheroid volume for the whole plate. This panel can be used to determine plate uniformity and the reproducibility of seeding the same

number of cells in each well. The panel is useful before drug additions (days in vitro 1-4) and can identify patterns of uneven cell seeding (drift, edge effects or other systematic sources of variability).

The bottom-right section of the spreadsheet (L14 to AF38) is used when multiple conditions are compared, for example in a dose-response assay. The template uses the first 6 replicates as the untreated control (used to normalise as 100%) and 0 for no spheroids (0% when normalised). It calculates the average and the median volumes for each condition as well as the Coefficient of Variation, normalised volumes and the area under the dose-response curve (AUC). The AUC is given as a percentage fraction of the whole dose-response area (the AUC if all conditions had 100% viability).

Panels 016 to T26 are colour coded red (larger) or blue (smaller) for spheroids deviating in volume with more than 2 standard deviations from the average volume for each condition.

Panels N29 to T39 show the relative deviation percentage from the mean for each condition, with values above 20% appearing as amber and above 30% as red. Both panels can be used to identify outliers and eliminate them from the analysis. The decision to eliminate outliers has to be based on documented data of the normal variation in size for the particular cell line and seeding procedure.

The drug concentrations in L16 to L26 can be changed to match the experiment in question. Untreated control wells are given a "virtual concentration value" 2 decimal logs (100 times) lower than the lowest drug concentration and complete kill "20% DMSO" wells are given a "virtual concentration value" of 2 decimal logs (100 times) higher than the highest drug concentration used. These virtual concentrations will not affect the results of the non-linear regression analysis as long as the curve fit is capped at the highest real drug concentration used.

20. In case of wells with missing spheroid or wells with multiple spheroids the number in "column A" would be different from the number in "(Series…)" in column B. For the correct operation of the template both numbers need to match.

21. If the plate reader has a temperature controlled chamber let it warm up to 37°C before the measurements. Alternatively, wrap the plates in aluminium foil and leave them to equilibrate to room temperature for 20 minutes before measuring fluorescence.

5. Acknowledgements

6. Supplementary files

The macro files and Excel spreadsheet are available through the Figshare database:

Macro 1 link: https://figshare.com/s/32f81784ee28e3fde015 (DOI: 10.6084/m9.figshare.3487919)

Macro 2 link: https://figshare.com/s/9952d072c3238a60e134 (DOI: 10.6084/m9.figshare.3487943)

Volume analysis template: https://figshare.com/s/6c57cede1d940f6fd952

(DOI: 10.6084/m9.figshare.3487940)

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Tables:

PBS wells											
Medium only	Untreated control (drug solvent)	0.01x**C**	0.03x**C**	0.1x**C**	0.3x**C**	Conc **C**=Reported IC50	3x**C**	10x**C**	30x**C**	100x**C**	25% DMSO (complete cell kill control)
PBS Wells											

Table 1. Example plate template including medium only (contamination) control, untreated

(negative or solvent) control and complete cell death (positive) control. The middle concentration **(C)**

can be chosen based on literature reported values or approximate potency within an order of

magnitude (e.g micromolar, nanomolar).