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TITLE:

Using High Content Imaging to Quantify Target Engagement in Adherent Cells

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Cellular thermal shift assay (CETSA), imaging, target engagement, p38α, high-throughput screening, single cell resolution

SUMMARY:

Measurements of drug target engagement are central to effective drug development and chemical probe validation. Here, we detail a protocol for measuring drug-target engagement using high content imaging in a microplate-compatible adaption of the cellular thermal shift assay (CETSA).

ABSTRACT:

Quantitating the interaction of small molecules with their intended protein target is critical for drug development, target validation and chemical probe validation. Methods that measure this phenomenon without modification of the protein target or small molecule are particularly valuable though technically challenging. The cellular thermal shift assay (CETSA) is one technique to monitor target engagement in living cells. Here, we describe an adaptation of the original CETSA protocol, which allows for high throughput measurements while retaining subcellular localization at the single cell level. We believe this protocol offers important advances to the application of CETSA for in-depth characterization of compound-target interaction, especially in heterogeneous populations of cells.

INTRODUCTION:

When developing new drugs or chemical probes it is essential to couple the observed pharmacological effect or functional readout to measurements of target occupancy or

engagement in live cells[1-3]. These data are necessary both to ensure that the small molecule in fact reaches its desired target and to validate the biological hypothesis behind protein target selection[4,5]. Furthermore, during drug development, model systems of increasing complexity are used to select and corroborate a lead compound prior to clinical trials. To confirm translation of biology across these preclinical systems, methods for tracing drug-target engagement and accompanying biology throughout this development process are critical.

Drug-target engagement has traditionally been challenging to monitor in live cells with unfunctionalized small molecules and proteins, especially at the single-cell level with spatial resolution[6,7]. One recent method to observe the interaction between unmodified drugs and proteins in live cells is the cellular thermal shift assay (CETSA) in which ligand-induced stabilization of a native protein in response to a heat challenge is quantified[8-10]. This is accomplished by quantifying remaining soluble protein after exposure to a heat challenge. In the initial disclosure of CETSA, western blot was used for detection. To enable screening campaigns and hit triaging of larger compound collections, efforts to increase the throughput of CETSA experiments have lead to the development of several homogenous, microplate-based assays[10,11]. However, one limitation with these methods is that they are currently best suited to compound treatment in cell suspensions and the detection requires cell lysis, leading to loss of spatial information. CETSA can be applied experimentally either as a ligand-induced shift in thermal aggregation temperature (T_{agg}) at a single concentration of the small molecule or the ligand concentration necessary to stabilize the protein at a single temperature. The latter is termed isothermal dose response fingerprints (ITDRF) to signify the dependence of these measurements on the specific experimental conditions.

The goal of this protocol is to measure target engagement using CETSA in adherent cells by immunofluorescent (IF) antibody detection with high-content microscopy[12]. This procedure extends the original CETSA platform to allow for single-cell quantification of target engagement with conservation of subcellular localization. Notably, unlike many previous reports, in this procedure compound treatment is performed in live adherent cells without surface detachment or washing prior to the heat challenge, thus preserving the established binding equilibrium we aim to measure[13]. Currently, the method is validated for one target protein p38α (MAPK14) in several cell lines, and we hope that by sharing this procedure the technique can be applied broadly across the melting proteome. We anticipate that this protocol can be adapted throughout the drug development pipeline from screening, hit triaging through to monitoring of target engagement *in vivo*.

PROTOCOL:

1. Seeding of Cells

Note: For a general overview of the workflow see **Figure 1**. A detailed list of materials and reagents are available in the **Table of Materials**.

1.1. Prior to seeding of the cells, drill holes with a standard drill in the frame of black 384-well

imaging assay plates to avoid air bubbles being trapped under the plate later during the heating step. To avoid plastic particles entering the wells during this step and to maintain sterile conditions, seal the plates with an adhesive aluminum foil or cover the plate prior to drilling in a tissue culture hood. Typically, 3 holes with a diameter of 3.5 mm on each of side of the plate (short edge) suffice.

1.2. Prepare a laminar flow bench by cleaning with 70% ethanol. Following standard aseptic tissue culture techniques, remove media from cell flask or dish. Wash cells with 5-10 mL of phosphate-buffered saline (PBS) and then add 2 mL trypsin to the flask. Incubate the flask at 37 °C until the A-431 cells detach. Count the cells either using a haemocytometer or cell counter. Prepare a cell suspension of 50,000 cells/mL in culture medium.

1.3. Dispense 40 µL of cell suspension (giving a final cell density of 2000 cells per well) into each well of an assay plate using a bulk reagent dispenser or a multichannel pipet, depending on the scale of the experiment. Briefly move the plate from side-to-side to disperse cells evenly on the bottom of the plate.

1.4. To minimize plate-edge effects, allow the cells to settle at the bottom of the assay plate for 20 minutes at room temperature in the back of the laminar flow hood. Then, place the plate in a plastic container with damp paper towels to ensure a humid atmosphere. Prior to use, wipe the plastic container with 70% ethanol.

1.5. Incubate the box with the assay plate for 2-3 days at 37 °C and 5% CO_2 in a conventional humidified incubator. Monitor confluency of the cells until 50-75%, as assessed by visual inspection with a light microscope.

2. Compound Treatment

2.1. On the day of the experiment, aspirate the medium from each well using a plate washer. Place the plate on the plate washer and select the aspiration program. If a plate washer is not available, then the liquid can be removed by inverting the plate with a rapid hand twist over a waste tray or sink. Complete removal of liquid is essential for good performance. Any excess liquid is then removed by dabbing with paper towels.

2.2. Add 30 µL of compounds diluted to the appropriated concentration in cell culture medium using automated dispensing or a multichannel pipette depending on the scale of the experiment. Ensure to add a negative (DMSO) and positive (known ligand) control to several wells on each assay plate. Since this is a thermal shift assay, it is necessary that the compound concentration exceeds the dissociation constant to observe protein stabilization. Thus, a rough guideline for compound concentration is 50-100 times the IC_{50}, but more detail descriptions for compound

concentrations are found in the Discussion section.

Note: DMSO tolerability of the cell lines should be tested prior to the experiment.

2.3. Seal the compound-treated assay plate with a breathable plate seal and incubate at 37 °C and 5% CO_2 in a humidified incubator for 30 minutes.

3. Heat Challenge

3.1. First, set the water bath to the desired temperature. Note that the final temperature that is reached inside the wells of the assay plate can be different from the final temperature in the water bath. Investigate the offset beforehand with a dummy plate and thermocouple thermometer. It typically takes 30 minutes for the bath to stabilize at the desired temperature.

3.2. To verify that the desired temperature is reached in the wells of the assay plate during the heating step, prepare an unsealed dummy plate containing the same volume of medium as the assay plate.

3.3. Remove the assay plates from the incubator. Take off the breathable seal and re-seal the assay plate containing the compound-treated cells with a tight adhesive aluminum foil to ensure that no water will leak into the wells during the subsequent heating in the water bath. Ensure that the drilled holes in plate frame are accessible.

3.4. Place the assay plate and the dummy plate in the water bath with the bottom of the plate angled towards the water surface to force any remaining air out from under the plates.

3.5. Monitor the temperature inside the wells of the dummy plate using a thermocouple thermometer.

3.6. Heat the assay plate in the water bath for 3 minutes. Immediately transfer the assay and dummy plate to another water bath with room-tempered water to cool down for 5 minutes. The assay plate is now ready for further processing.

4. Fixation

4.1. Dispense 10 µL 16% (w/v) paraformaldehyde (PFA) directly to the assay plate using a bulk reagent dispenser or a multichannel pipet. Incubate at room temperature for 20 minutes.

Note: Some fixatives are classified as carcinogenic and institutional safety regulations should be followed.

4.2. Aspirate the PFA solution and wash the cells with 300 µL PBS using a plate washer. Place the plate on the plate washer and select the aspiration program.

Note: This procedure has been optimized using an overflow protocol on the plate washer in which liquid is simultaneously dispensed and removed. If a plate washer or similar procedure is not available, the washing step can alternatively be done manually.

4.3. *Manual washing procedure:* Remove the liquid from the wells by inverting the plate with a rapid hand twist over a waste tray or sink. Complete removal of liquid is essential for good performance. Add 80 µL of PBS with a multichannel pipette and invert the plate again as described above, repeat the washing procedure two times. After the last wash, blot the plate against clean paper towels to remove any excess liquid.

5. Permeabilization

5.1. Add 20 µL of 0.1% (v/v) NP-40 to the wells with a multichannel pipet and incubate at room temperature for 10 minutes. Wash the cells using the same procedure as described above (step 4.2).

5.2. Alternatively, when appropriate, apply an antigen retrieval protocol, *e.g.*:

5.2.1. Add 80 µL of 10 mM glycine at pH 7.2 and incubate for 10 minutes at room temperature. Aspirate the glycine solution using a plate washer by placing on the plate washer and selecting the aspiration protocol. See notes in 2.1 and 4.2 if a plate washer is not available.

5.2.2. Add 20 µL of 1% SDS to the wells with a multichannel pipet. Incubate at room temperature for 5 minutes and wash according to the same procedure described above (step 4.2).

6. Blocking

6.1. Add 15 µL of 1% (w/v) bovine serum albumin (BSA) in PBS to the wells using a bulk reagent dispenser or a multichannel pipet. Incubate the plate at room temperature for 1 hour or overnight at 4 °C with an aluminum foil plate seal.

7. Primary Antibody

7.1. Aspirate the blocking solution using a plate washer by placing on the plate washer and selecting the aspiration protocol. See notes in 2.1 and 4.2 if a plate washer is not available.

7.2. Add 10 µL of primary antibody diluted accordingly in 1% (w/v) BSA in PBS to the wells using a multichannel pipet. Incubate the plate at room temperature for 1 hour or overnight at 4 °C with an aluminum foil plate seal.

8. Secondary Antibody

8.1. Aspirate the primary antibody solution and wash the wells according to same procedure as

described above (step 4.2).

8.2. Add 10 µL of Alexa 488 secondary antibody diluted accordingly in 1% (w/v) BSA in PBS. Incubate at room temperature for 1 hour. Seal the plate with an adhesive aluminum foil seal to protect from light.

Note: Protect the plate from light during this and subsequent steps.

9. Nuclear Staining and Cell Mask

9.1. Add 10 µL of nuclear dye diluted to 0.05 mg/mL in PBS to the wells using a multichannel pipet. Incubate at room temperature for additional 10 minutes.

9.2. Aspirate the secondary antibody and Hoechst solution and wash the wells using the same procedure as described above (step 4.2).

9.3. Add 10 µL of cell mask diluted to 200 ng/mL in PBS to the wells using a multichannel pipet. Incubate at room temperature for 30 minutes.

9.4. Aspirate the cell mask solution and wash the wells using the same procedure as described above (step 4.2).

9.5. Dispense 60 µL of PBS to all wells using the plate washer, a bulk reagent dispenser, or a multichannel pipet, and seal the plates with an adhesive aluminum foil.

10. Image Acquisition and Analysis

10.1. Capture images on a high content imager using 3 fluorescent channels: DAPI (387/447), GFP (472/520), and TexasRed (562/624). Acquire 4 images per well using 10X objective. Use automated laser autofocus and apply binning 2 during acquisition. Store images as 16 bit, gray scale tiff files along with metadata.

10.2. Analyze images using available software. Identify cell boundaries using a Cell Scoring algorithm with DAPI (nucleus) and TexasRed (cytoplasm).

10.3. Extract average intensity for all acquired wavelengths for further data analysis.

10.4. Calculate the Z-factor to ensure the robustness of the assay.

10.5. Calculate % stabilization using the following formula: 100*(1-(well intensity – average well intensity negative control)/(average intensity positive control-average intensity negative control)). Here negative control is DMSO and positive control is the reference substance. Since the maximum stabilization between compounds can vary and in fact be greater than the positive control for ITDRF curves, the maximum and minimum stabilization intensities for each compound

are sometimes used in place of the intensity values of the control wells.

REPRESENTATIVE RESULTS:

The protocol outlined in **Figure 1** describes the basic workflow for running CETSA assays on adherent cells with detection of remaining soluble protein by high content imaging. This workflow can be easily adapted to all stages of assay development by modifying the plate layout of the compounds or reagents[14]. We detail expected results for several anticipated use cases below.

Antibody identification and assay development. A prerequisite for successful results is the identification of a primary antibody or other suitable affinity reagent that selectively recognizes the native form of the protein in the presence of the aggregated and precipitated protein formed during the heat challenge in step 3. To establish the CETSA imaging assay described here, we screened a panel of 9 antibodies targeting p38α at 52°C for signal window between the positive and negative controls. We then titrated the best antibodies and settled on the conditions shown in **Figure 2 A,B** with representative immunofluorescence images for p38α stabilized by a known ligand (positive control) and DMSO (negative control). Antibody recognition should also not be disrupted by the conformational changes of the target protein that may be induced by ligand binding (**Figure 2C**). As an example, BIRB796 has a long off rate, and quantification of target engagement was only possible by applying an antigen retrieval step (5.2; **Figure 2D**). It is important to validate the performance of the primary antibody with known ligands covering different binding sites of the target protein if available. The antibody validation is preferably done both with and without the antigen retrieval step.

T_{agg} and ITDRF curves. As mentioned above, CETSA experiments can be run in two different modes, T_{agg} curves and ITDRF experiments. Both variants utilize the same basic protocol outlined in **Figure 1** and in the protocol section. In the first setup, the purpose is to challenge the cells with a temperature gradient and compare the T_{agg} curves in the presence and absence of a single concentration of ligand. To perform a T_{agg} curve, separate plates are heated for 3 minutes across a range of temperatures. In performing this experiment, it is important to time the compound treatment length for each plate with the time it takes for the water bath to stabilize to the new temperature. In this regard, peforming the heat challenge step in the water bath is more time consuming than heating tubes in a PCR machine. The experimental path is to next run concentration response curves of a ligand at a fixed temperature to generate ITDRF curves. In general, when testing multiple compounds, assay ready plates for the compound addition are prepared using automated liquid handling to achieve the most reproducible data. Compounds are serially diluted in DMSO and then dissolved in cell culture media to the desired concentrations. We have typically tested 11 point concentration series starting at 50 – 100 µM in 3 or 4 fold dilution, but this depends on the potency of the ligands used. It is advised to first establish the T_{agg} curve both in absence and presence of a ligand and select the temperature for subsequent isothermal experiments where a shift between the curves can be observed. The selected temperature should be around or just above the T_{agg}. Both formats allow for confirmation of target engagement but for ranking of compound affinities ITDRF experiments are often more suitable. **Figure 3A** shows an illustration of anticipated quantified results for a T_{agg}

curve and **Figure 3B** quantifies results of a typical ITDRF experiment.

Screening campaign. The protocol can also be adapted to screening campaigns to identify novel binders of the target protein. In this case, isothermal heat challenges are applied for a large number of compounds at a single concentration followed by ITDRF experiments for identified stabilizing compounds. We prepare assay ready screening plates and transfer the compounds diluted in culture media to the assay plates using automated liquid handling. As with all thermal stability assays, it is necessary to exceed the dissociation constant to observe protein stabilization, and thus we have applied small molecule libraries at 50 µM to facilitate hit identification. Triaging of the hits using ITDRF will later allow ranking and prioritization of these compounds. **Figure 4** shows a representative result from a screening plate.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic overview of the protocol described in this article.

Figure 2. Antibody identification and assay development. A) Example data for detection of human p38α in A-431 cells. Representative images of positive (1 µM AMG548) and negative (DMSO) controls. Red- nuclear Hoechst staining, Green- p38α staining. Images taken with 10X magnification; the white scale bar represents 73 µm. B) Example of quantified data expressed as average intensity/cell. Error bars represent standard error of mean of 16 replicates for 6 separate plates. Each plate was heated to 52 °C in a water bath followed by fixation of the cells, permeabilization and subsequent immunostaining. C) Immunofluorescence signal intensity measured after treating A-431 cells with 1 µM BIRB796 or DMSO as described above followed directly by fixation. In the absence of a heating step, the BIRB796 signal is lower than the DMSO signal, suggesting that BIRB796 disrupts the detection of p38α with this antibody. D) $ITDRF_{CETSA}$ curves for cells treated with BIRB796 either with (blue triangle) or without (grey triangle) antigen retrieval protocol. This figure has been modified from Axelsson *et al.* 2018[12]. Copyright 2018 American Chemical Society.

Figure 3. Thermal aggregation and ITDRF experiments. A) Thermal aggregation curve experiments for cells treated with positive (1 µM AMG548, in orange) and negative (DMSO in grey) controls. Error bars represent standard error of mean of 32 or 464 replicates. B) $ITDRF_{CETSA}$ experiment performed at 52 °C for cells treated with serial dilutions of SB203580 in blue, Skepinone-L in green and RWJ67657 in red. Error bars represent standard error of the mean of 6 replicates. Quantified data are expressed as average intensity/cell and normalized against the highest concentration of respective compound. This figure has been modified from Axelsson *et al.* 2018[12]. Copyright 2018 American Chemical Society.

Figure 4. Representative overview of a screening plate at 10X magnification. Positive controls (1 µM AMG548) are in columns 2 and 24 and negative controls (DMSO) are in columns 1 and 23. All other wells contain 50 µM of the library compounds used for screening with several hits denoted. In the inset figures, the white line in the lower right corner represents 200 µm. This figure has been modified from Axelsson *et al.* 2018[12]. Copyright 2018 American Chemical Society.

DISCUSSION:

As discussed in the results section, there are several key steps to the procedure. First, it is important to identify a high-quality affinity reagent. We recommend screening a small library of antibodies for each desired target. After a primary antibody has been selected, it is also important to validate the system for a number of different binding sites of the protein target if appropriate. Counter-screening for compounds that interfere with the assay signal as shown in **Figure 2C** by omitting the heat challenge is highly encouraged after a screening campaign. When a reduced signal in presence of a ligand is observed, an antigen retrieval protocol as described in step 5.2 can be tested. Uneven heating of the plates can cause fluctuations and plate-to-plate variations in the observed data. Since the plates are partly submerged in a water bath during the transient heat challenge (step 3.4) the outer wells of the plates are exposed to the hot water not only at the bottom of the wells but also through the outer wall. This may cause higher temperature during the same submersion time in the outer wells of the plate and subsequent plate edge effects. Therefore, it is recommended to avoid the outer wells of the plate. Similar effects can also be seen if air bubbles are trapped under the plate during the heating step preventing sufficient contact between the hot water and the bottom of the wells. Given the challenges with heating imaging plates, devices and plates specifically made for exposure to heat could help advance this method. We have explored the use of several cell lines, and have found variability in surface attachment after the heat challenge for adherent cell types. Preliminary experiments in our lab suggest that the use of a coating or extracellular matrix such as Synthemax II-SC or Cell-Tak can minimize cell detachment, but this could pose a technical challenge when using semi-attached cell types. Since compound treatment is performed in live cells, the small molecules must be cell permeable. If the compound is not membrane permeable, alternative high throughput CETSA methods are recommended. Some compounds need to be metabolically activated to bind to their target proteins[11]. In such cases, longer treatment cells with the compound prior to the heat challenge is recommended.

This protocol requires a single plate from cell seeding to imaging making it amenable to high throughput screening campaigns. The number of cells for each experiment is much lower than can be achieved with western blots or previous AlphaScreen assays[15]. Furthermore, washing or cell detachment steps, which may alter compound availability and binding, are removed, preserving the established binding equilibrium through the heat challenge step. Unlike previous procedures, a lack of signal due to cytotoxicity is simultaneously reported on by nuclear staining. Finally, imaging preserves the spatial resolution of the individual cells, allowing for target engagement measurements in mixed populations of cells or cell states.

To truly assess the applicability of the approach, concerted efforts to apply the technique across the melting proteome are needed. Such experiments will clarify the ease of identifying suitable affinity reagents, which report selectively on the native protein in the presence of the remaining denatured and aggregated proteins. We currently do not know how protein expression levels will affect the ability to reliably measure a signal window. We expect this to reflect affinity and selectivity of the available antibodies, and anticipate that for low abundant proteins technologies used to enhance signal in immunofluorescent assays will be applicable. Alternatively, tagged proteins or functionalized compounds could be used for detection in modified versions of the

described protocol. This protocol is limited to targets that melt and for interactions where a quantifiable stabilization can be observed. For example, endogenous ligands can stabilize a protein in live cell assays, which may prevent further stabilization by an exogenous ligand. Although not explored here, we believe that it will be possible to multiplex readouts to allow for the study of multiple targets simultaneously, or a target with downstream effectors. Further studies are necessary to investigate these aspects of *in situ* CETSA.

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DISCLOSURES:

The authors have no disclosures to report.

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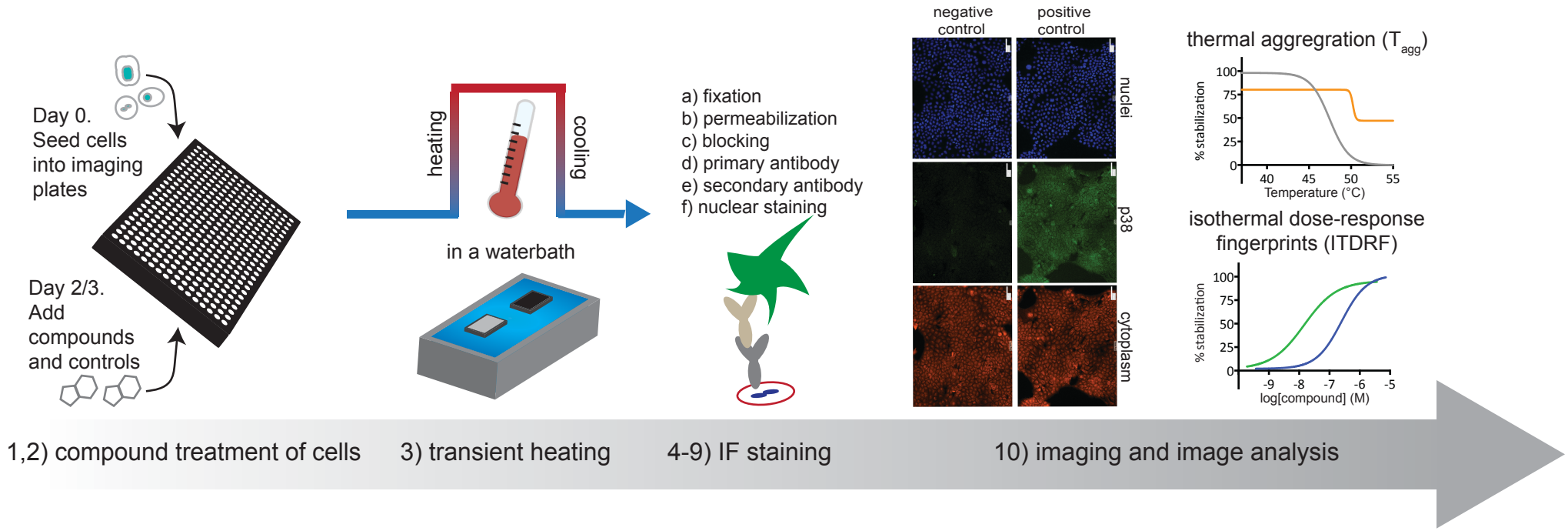
Figure 1



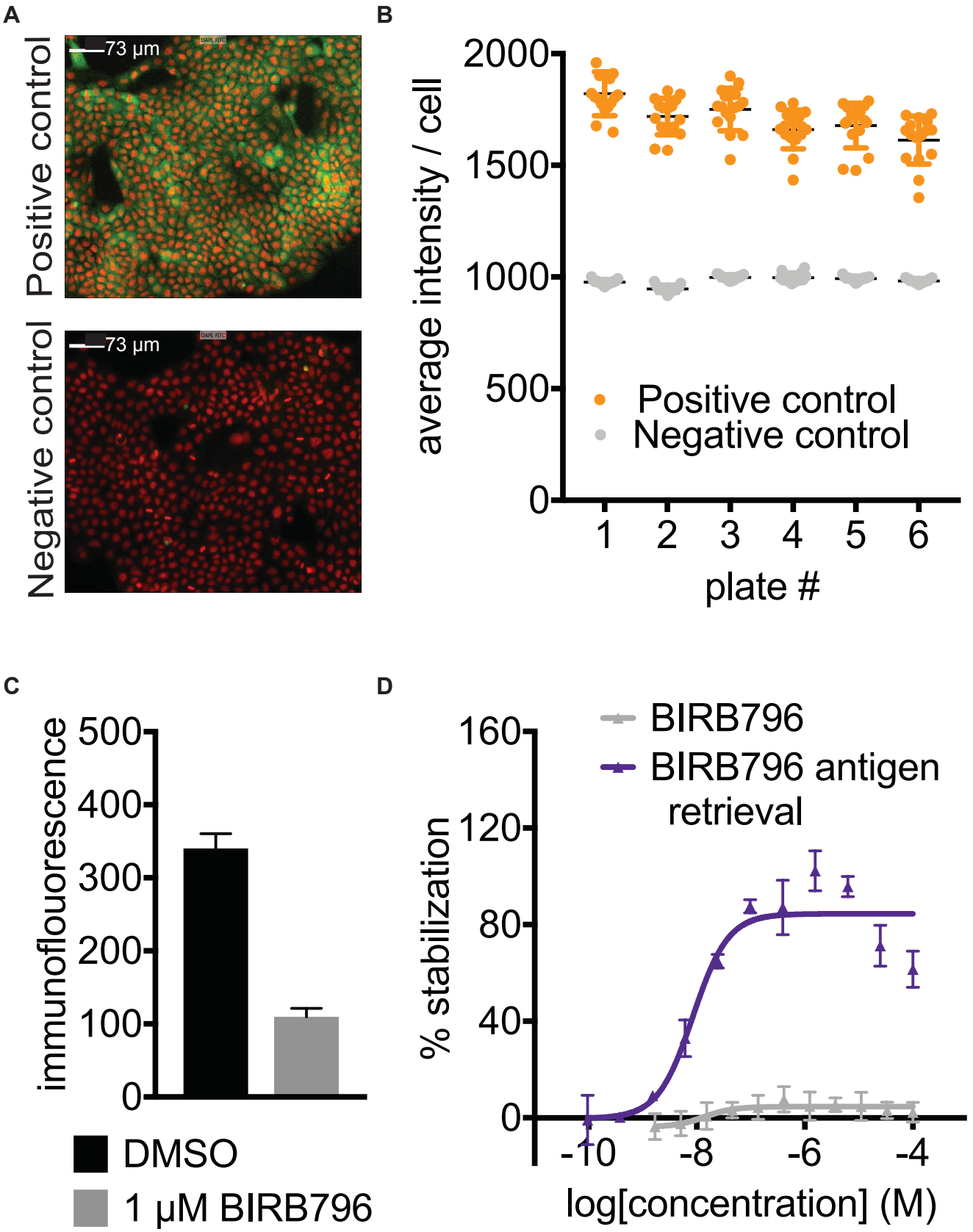
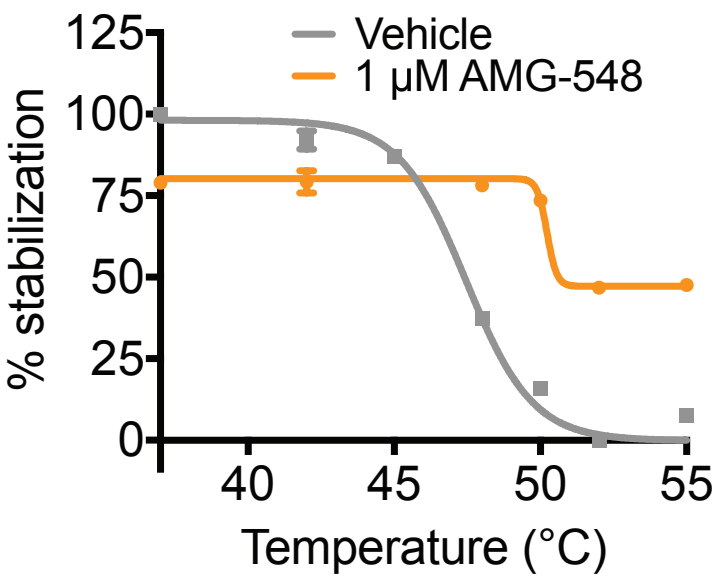


Figure 3

A



B

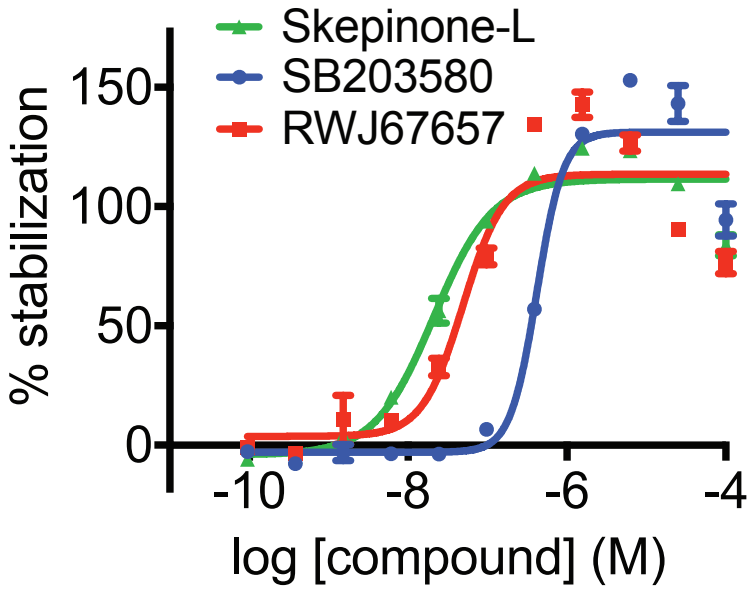
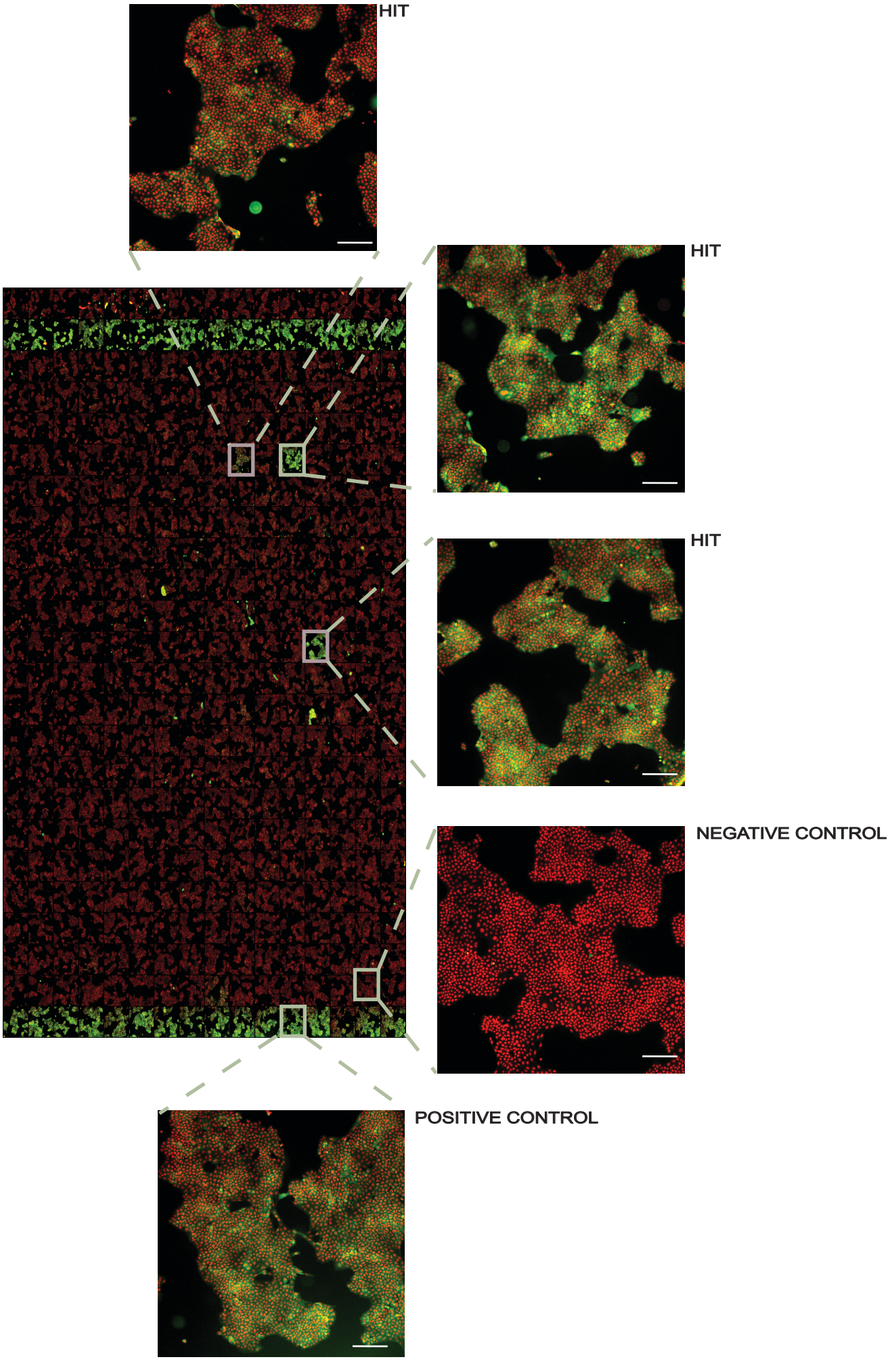


Figure 4



Name of Material/ Equipment	Company	Catalog Number
Phosphate-buffered saline (PBS)	Medicago	09-9400-100
TrypLE Express	ThermoFisher Scientific	12604013
16% paraformaldehyde (PFA)	ThermoFisher Scientific	28908
Goat anti-rabbit IgG (H+L), Alexa Fluor 488 conjugated antibody	ThermoFisher Scientific	A11008
HCS CellMask Red stain	ThermoFisher Scientific	H32712
NP-40	Sigma-Aldrich	56741
Hoechst stain 33342	Sigma-Aldrich	B2261
Dulbecco's modified Eagle's medium (DMEM) - high Glucose	Sigma-Aldrich	6429
Heat-inactivated fetal bovine serum (FBS)	Sigma-Aldrich	F9665
Penicillin-Streptomycin	Sigma-Aldrich	P4333
Corning, breathable plate seal	Sigma-Aldrich	CLS3345
Rabbit anti-p38 antibody [E229]	Abcam	ab170099
Falcon, Black 384-well clear bottom imaging plates	VWR	736-2044
Greiner, 384-well low volume polypropylene plates	VWR	784201
Adhesive aluminum foil	VWR	30127790
Peelable aluminium seal	Agilent	24210-001
LY2228820	Selleckchem	S1494
PH797804	Selleckchem	PH797804
BIRB796	Selleckchem	S1574
SB203580	Tocris	1202
AMG 548	Tocris	3920
RWJ 67657	Tocris	2999
L-Skepinone	CBCS compound collection	
Bovine serum albumin (BSA)	Sigma-Aldrich	A7030
SDS (sodium dodecyl sulfate)	BDH	44244
Glycine	Sigma-Aldrich	G8898
A-431 cells	ATCC	ATC-CRL-1555
Echo 550	Labcyte	
Plate sealer	Agilent	
Bulk reagent dispenser	Thermo Scientific	5840300

Automated liquid handling	Agilent	
Plate washer	Tecan	
Water bath	Julabo	TW12
Thermocouple	VWR	
High content imager	Molecular Devices	

Comments/Description

for detaching cells and subculturing
fixative

secondary antibody
Cytoplasm stain
for permeabilization
nuclear stain

cell culture media component
cell culture media component
cell culture media component
for copound incubation step
primary antibody, LOT:GR305364-16
imaging plates

for PlateLoc
p38α inhibitor
p38α inhibitor
p38α inhibitor
p38α inhibitor
p38α inhibitor
p38α inhibitor
p38α inhibitor
blocking agent
used in antigen retrieval
used in antigen retrieval

For preparation of compound plates
PlateLoc
Multidrop Combi

Bravo liquid handling platform; used for compound plate preparation
Hydrospeed

Thermocouple traceable lab thermometer
ImageXpress Micro XLS Widefield High-Content Analysis System

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Title of Article: Using high content imaging to quantify target engagement in adherent cells

Author(s): Hanna Axelsson, Helena Almqvist, and Brinton Seashore-Ludlow

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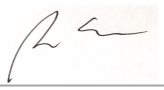
Signature:	
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Stockholm, Sweden
August 14, 2018

To the review editor,

Please find our revised manuscript JoVE58670, "Using high content imaging to quantify target engagement in adherent cells". We appreciate the constructive comments from both the reviewers and editors and have revised our manuscript to address these points. In the attached response file you will find the detailed adjustments we have made to the manuscript files and figures in order to thoroughly answer these questions. We believe that our clarifications, editing and experiments satisfactorily address the concerns posed by the reviewers and hope that the manuscript is now suitable for publication in *JoVE.*

Thank you for your time and consideration.
Best regards,
Brinton Seashore-Ludlow

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have taken time to proofread the manuscript.

2. Please revise lines 325-327 and 331-333 to avoid previously published text.

Thank you for pointing this out. We have changed these lines.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have uploaded a copyright page from ACS regarding figures 2-4. Figure 1 is original to this work.

4. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.

All figures have been uploaded as .ai files.

5. Figures 2 and 3: Please line up the panels better. Some panels are off-set in Figure. Please ensure that the panels are of the same dimensions if possible.

We have re-sized and aligned the panels in Figures 2 and 3 accordingly.

6. Figures 2 and 4: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

We have commented on the magnification used in the figure legend and added scale bars to these figures.

7. Affiliation 3 is not assigned to any author. Please check.

Thank you for pointing this out and the extra affiliation has been removed.

8. Please spell out each abbreviation the first time it is used.

We have spelled out each abbreviation in the revised text.

9. 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 and Reagents. For example: Multidrop, Traceable, ImageXpress, Molecular Devices, MetaXpress, etc.

In the revised manuscript we have removed commercial product names for instruments and reagents and have these listed in the Table of Materials and Reagents.

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised our protocol text to reflect this request where applicable.

11. Lines 85-86: This note may be deleted because such information is mentioned in step 1.1.

Lines 85-86 have been removed from the text.

12. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

We have adjusted the language our protocol accordingly.

13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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.

We have added significant detail to our protocol and hope the “how” for these steps is now clear.

14. 1.1: Please specify the type and size of assay plates. What is used to drill holes? How large is the holes? How many holes are drilled per plate?'

We have added more detail to the instructions for this step to clarify these points.

15. 1.2: Please specify the conditions used to trypsinize the cells.

We have added instructions for cell trypinization.

16. 2.2: What concentration is considered to be appropriate?

We have added discussion and instructions on compound concentrations.

17. Lines 252-262: Please move this paragraph to the Discussion section.

This paragraph has been moved to the discussion section.

18. Discussion: Please also discuss any limitations of the technique.

We have added several limitations including those brought up by the other reviewers.

19. References: Please do not abbreviate journal titles.

The reference section has been updated accordingly.

20. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

The lot number of the antibody has been added to the Table.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript entitled "Using high content imaging to quantify target engagement in adherent cells" by Axelsson, Almqvist and Seashore-Ludlow describes the detailed steps of an adaptation of the previously reported CETSA format. The original paper with the data presented again here was a well needed adaptation of the CETSA protocol, allowing also studies of adherent cells without prior detachment. The potential to study adherent cells without detachment prior to detection is intriguing as the actual detachment could affect the cellular uptake and intracellular biology. Having a detailed protocol will certainly help spreading the use of the adaptation.
The protocol is not too detailed but well enough for researchers with previous understanding of cell staining and fixation.

Major Concerns:
Rows 120-121 and 236-238. How does one construct a Tagg curve in a waterbath? This may be worth describing in better detail as it is not apparent from the protocol how that could be carried out. Again, this raises the question on how to approach targets with smaller shifts than p38a where the transfer from Tagg to ITDFRs is less forgiving?

We have added detail in the representative results section on how to create a Tagg curve. We have also added the limitation of targets with small shifts to the discussion section.

Minor Concerns:
A few specific comments come to mind when reading the manuscript:
1) There is an affiliation not belonging to neither of the authors in the author list (row 13)

See above

2) The statement on rows 62-63 could be clarified to underline that it is only the detection step that has required lysis, not that the incubation and heating steps of the original CETSA protocol requires lysed cell suspensions. In fact, the presented protocol also requires the permeabilization of cells to allow affinity reagents to reach the antigen,

making it a somewhat lysed suspension. This is semantics.

We have tried to clarify these steps in the introduction section.

3) Row 73, something missing

We have re-written this sentence.

4) Rows 75-77. The study behind this protocol has already been published but maybe there could be room also in this detailed protocol for a discussion on how to approach targets with smaller shifts than p38a. Will there be challenges?

This has been addressed in the discussion section.

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5) Row 86 and 90. The drilling of holes in plates does not resonate well with HTS screening. Are there no plates available that minimize the amount of air trapped? On that same note, regarding the edge effects (rows 254-255) - there are plates called "edge plates" with a reservoir surrounding the outer wells. Would such plates, without filling up the edge reservior minimize the amount of trapped air and at the same time reduce the heating surface of the edge wells?

Interesting idea with reservoir plates and though applicable to cell culture, we are not aware of reservoir plates for imaging purposes. We feel that though these may address the edge wells, they will not influence air trapped under the plate.

6) Row 111, please add an alternative for less equipped labs, similar to the step of dispensing PFA with a multidrop or multichannel pipet.

We have added an alternative procedure.

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7) Row 260, variable?

We have fixed this sentence.

8) 291 and Figure 5. I do not see the need for this figure. Those reading this protocol should know how edge effects display.

We have removed this figure.

9) In the discussion part the authors discuss the need for good antibodies, this is of course imperative. Could this be a limiting factor for new targets where antibody availability is scarce (and where early screens are carried out). Could the use of a tagged protein be helpful?

We have updated the discussion text to address this point.

Reviewer #2:
Manuscript Summary:
The article entitled "Using high content imaging to quantify target engagement in adherent cells" proposes a new adaptation of the CETSA assay, in order to characterize

deeply the interaction between a compound and its putative target. It is based on the high throughput measurements of drug-target interaction by using a specific antibody against the putative target followed by the incubation of a fluorescent secondary antibody. Then the interaction between the compound and its target is analysed by using a microplate-reader that permit a high throughput analysis. The new CETSA method is very interesting and offers important advances for a rapid screening of a library of new compounds especially if it is a big library.

Major Concerns:
Figure 1D .Could be interesting for the readers to see a comparison between a normal CETSA result (western blot analysis) and this new CETSA method, also for a single compound, to visualize the results between of both methods and evaluate if the sensitivity of the two methods is equal or different.

We have data in the lab examining CETSA results for A-431 cells in suspension using both western blot and AlphaScreen technologies. These compare well. We have also published similar comparison data for K562 and HL-60 in reference 10 and compared AlphaScreen to the imaging assay in reference 12. Notably, comparisons between assay formats are challenging when the heating format is not the same, as the exact heating time of the sample for example in a water bath and in a PCR tube differs. In such comparisons right shifting of the potency for the sample that was heated to the temperature farthest from the T_{agg} is observed. Thus, for the true comparison we would want to compare adherent A-431 in imaging plates as detected by imaging and western blot. This poses a technical challenge as the amount of protein necessary for the western blot is difficult to obtain in the 384-plate format. Thus, we refer the reviewer to the already published comparisons.

Minor Concerns:
Authors should discuss some issues listed below:
Step 2.3: This step proposes to incubate cells with the different compounds for 30 minutes at 37°C, but if the main goal of the method is to screen library of new compounds, how the Authors may exclude that the lack of interaction is not due to a reduced entry of the compound into the cells? In other terms they have never shown that a negative compound remain negative also after longer incubation.

The length of compound treatment varies and certain molecules that need metabolic activation will need a longer incubation time. We have addressed this in the revised text.

Steps 4.2/8.1/9.2/9.4: How many times did you wash the wells with PBS? Please provide the number of wash in this step since for our knowledge the 384 wells can maximum contain 100µL of a liquid. Therefore they mean 3 washes of 100µL or each wash with 300 µL, and if this is the case, how many washes are sufficient to further proceed in the method? Please add more details.

We have provided more details for these steps to clarify these points in the protocol.

Step 5.2: In this step Authors have written: "Alternatively, when appropriate, apply an antigen retrieval protocol…". According to your original protocol published in ACS Chemical Biology, the antigen retrieval step is useful for minimizing the number of false negative because it facilitates the detection of the target engagement. How new users

can define when the antigen retrieval protocol is necessary? May the Authors give more hints about this important decision-step?

We believe this should be used as a counter-screening method and if target engagement is not observed for known binders. We have clarified this in the text.

Reviewer #3:
Manuscript Summary:
The authors present a short, but well described protocol to perform an exciting new technique. The manuscript is well written and should serve as a great resource for the research field. The description of antibody limitations is quite helpful.

Major Concerns:
Perhaps the authors could expand upon the calculation of % stabilization from the raw data. It is difficult to follow how that relates to fluorescence intensity and thus why the drug treated curve in Figure 3A starts at 75% and ends at 50% while the control spans 100 - 0 %.

We have added this to the protocol steps.

Minor Concerns:
In Figure 5, the lookup table labels need to be fixed.

This figure has been removed.

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Title: In Situ Target Engagement Studies in Adherent Cells

Author: Hanna Axelsson, Helena Almqvist, Magdalena Otrocka, et al

Publication: ACS Chemical Biology

Publisher: American Chemical Society

Date: Apr 1, 2018

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TITLE:

Using high content imaging to quantify target engagement in adherent cells

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KEYWORDS:

CETSA, imaging, target engagement, p38α, HTS, single cell resolution

SUMMARY:

Measurements of drug target engagement are central to effective drug development and chemical probe validation. Here, we detail a protocol for measuring drug-target engagement using high content imaging in a microplate-compatible adaption of the cellular thermal shift assay (CETSA).

ABSTRACT:

Quantitating the interaction of small molecules with their intended protein target is critical for drug development, target validation and chemical probe validation. Methods that measure this phenomenon without modification of the protein target or small molecule are particularly valuable though technically challenging. The cellular thermal shift assay (CETSA) is one technique to monitor target engagement in living cells. Here, we describe an adaptation of the original CETSA protocol, which allows for high throughput measurements while retaining subcellular localization at the single cell level. We believe this protocol offers important advances to the application of CETSA for in-depth characterization of compound-target interaction, especially in heterogeneous populations of cells.

INTRODUCTION:

When developing new drugs or chemical probes it is essential to couple the observed pharmacological effect or functional readout to measurements of target occupancy or engagement in live cells[1-3]. These data are necessary both to ensure that the small molecule in fact reaches its desired target and to validate the biological hypothesis behind protein target selection[4,5]. Furthermore, during drug development model systems of increasing complexity are used to select and corroborate a lead compound prior to clinical trials. To confirm translation of biology across these preclinical systems methods for tracing drug-target engagement and accompanying biology throughout this development process are critical.

Drug-target engagement has traditionally been challenging to monitor in live cells with unfunctionalized ~~compounds~~ small molecules and ~~native~~ proteins, especially at the single-cell level with spatial resolution[6,7]. One recent method to observe the interaction between unmodified drugs and proteins in live cells is the cellular thermal shift assay (CETSA) in which ligand-induced stabilization of a native protein in response to a heat challenge is quantified[8-10]. This is accomplished by ~~detecting~~ quantifying ~~the~~ remaining soluble protein after ~~the~~ exposure to a heat challenge. ~~and~~ In ~~in~~ the initial ~~protocol~~ disclosure of CETSA w~~was initially reported using western blot~~W~~estern blot was used for detection. To enable screening campaigns and hit triaging of larger compound collections e~~E~~fforts to increase the throughput of CETSA experiments have lead to the development of several homogenous, microplate-based assays[10,11]. However, one limitation with these methods is that they are currently ~~limited~~ best suited to compound treatment in cell suspensions and the~~to~~ ~~measurements~~ detection requires cell lysis leading to loss of spatial information~~in lysed cell suspensions~~. CETSA can be applied experimentally either as a ligand-induced shift in thermal aggregation temperature (T_{agg}) at a single concentration of the small molecule or the ligand concentration necessary to stabilize the protein at a single temperature. The latter is termed isothermal dose response fingerprints (ITDRF) to signify the dependence of these measurements on the specific experimental conditions.

The goal of this protocol is to measure target engagement using CETSA ~~in live,~~ in adherent cells by immunofluorescent (IF) antibody detection with high-content microscopy[12]. This procedure extends the original CETSA platform to allow for single-cell quantification of target engagement with conservation of subcellular localization ~~in~~. Notably, unlike many previous reports, in this procedure compound treatment is performed in live adherent cells without surface~~removes the need to~~ detachment or washing ~~adherent cells~~ prior to the heat challenge, thus preserving the established binding equilibrium~~m~~ we aim to measure ~~through the heat challenge step~~[13]. Currently, the method is validated for one target protein p38α (MAPK14) in several cell lines and we hope that by sharing this ~~protocol~~ procedure the technique can be applied broadly across the melting proteome. We anticipate that this protocol can ~~easily~~ be adapted throughout the drug development pipeline from screening, hit triaging through to monitoring of target engagement *in vivo*.

PROTOCOL:

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For a general overview of the workflow see **Figure 1**. A detailed list of materials and reagents are available in the Table of Materials.

~~Note: During the heating step, it is critical to avoid air bubbles trapped under the assay plate. To prevent this, holes can be drilled in the frame of the assay plates prior to seeding of the cells.~~

1. Seeding of cells

~~1.1.~~ Prior to seeding of the cells, drill ~~3.5 mm~~ holes with a ~~drilling machine~~standard drill in the frame of black 384 ~~-~~well ~~the~~ imaging assay plates to avoid air bubbles being trapped under the plate later during the heating step. To avoid plastic particles entering the wells during this step and to maintain sterile conditions, seal the plates with an adhesive aluminum foil or cover the plate prior to drilling in a tissue culture hood. ~~.~~ Typically 3 ~~holes with~~holes with a diameter of 3.5 mm on each of side of the plate (short edge) suffice.

1.1.

1.2. Prepare a laminar flow bench by cleaning with 70% ethanol. Following standard ~~aspetic~~aseptic tissue culture techniques ~~In a tissue culture hood~~ remove media from cell flask or dish. Wash cells with 5-10 mL phosphate-buffered saline (PBS) and then add 2 mL ~~t~~Trypsinizetrypsin to the flask. Incubate the flask at 37 °C until the A-431 cells detach. ~~and count~~Count the ~~A431~~A-431 cells either using a haemocytometer or cell counter. Prepare a cell suspension of 50 000 cells/ml in culture medium.

1.3. Dispense 40 µL of cell suspension (giving a final cell density of 2000 cells per well) into each well of an ~~384-well imaging microtiter~~assay plate~~. We recommend~~ using a ~~Multidrop~~ bulk reagent dispenser or a multichannel pipet depending on the scale of the experiments~~.~~ Briefly move the plate from side-to-side to disperse cells evenly on the bottom of the plate.~~.~~

1.4. To minimize plate ~~-~~edge effects, allow the cells to settle at the bottom of the assay plate~~s~~ for 20 minutes at room temperature in the back of the laminar flow hood. Then place the plate~~s~~ in a plastic container with damp ~~cloths~~ paper towels to ensure a humid atmosphere. Prior to use wipe the plastic container ~~should be wiped down~~ with 70% ethanol.

1.5. Incubate the box with the assay plate~~s~~ for 2-3 days at 37 °C and 5% CO_2 in a conventional humidified incubator. Monitor ~~C~~confluency of the cells ~~should be monitored and~~ until 50-~~70~~75% ~~confluency~~ as assessed by visual inspection with a light microscope.

~~1.5. is recommended depending on cell type.~~

2. Compound treatment

2.1. On the day of the experiment aspirate the ~~medium~~ medium ~~to~~from each well using a plate washer. Place the plate on the plate washer and select the aspiration program. If a plate washer is not available then the liquid can be removed by inverting the plate with a rapid hand twist

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over a waste tray or sink. Complete removal of liquid is essential for good performance~~, by turning the plate upside down and quickly flicking the plate~~. Any excess liquid is then removed by dabbing with paper towels.

2.2. Add 30 µL of compounds diluted to the appropriated concentration in cell culture medium using automated dispensing or a multichannel ~~pipet~~pipette depending on the scale of the experiment. Ensure to add a negative (~~vehicle~~DMSO) and positive (known ~~stabilizing compound~~ligand) control to several wells on each assay plate. Since this is a thermal shift assay it is necessary that the compound concentration ~~to~~ exceeds the dissociation constant to observe protein stabilization. Thus a rough guideline for compound concentration is 50-100 times the IC_{50}, but more detail descriptions ~~Guidelines~~ for compound concentrations are found in the Discussion section. Note: Do not exceed concentrations of 0.1% ~~or higher of~~ DMSO when performing compound treatments on live cells unless assess prior to the experiment.~~.~~

2.3. Seal the compound-treated assay plate~~s~~ with a breathable plate seal and incubate at 37 °C and 5% CO_2 in a humidified incubator for 30 minutes.

3. ~~Heating of cells~~Heat challenge

3.1. First set the ~~temperature of a~~ water bath to the desired temperature. Note~~:~~ that the final temperature that is reached inside the wells of the assay plate can be different from the final temperature in the water bath. Investigate the offset beforehand with a dummy plate and thermocouple thermometer. It typically takes 30 minutes for the bath to stabilize at the desired temperature.

3.2. To verify that the desired temperature is reached in the wells of the assay plate~~s~~ during the heating step, prepare an unsealed ~~control~~ dummy plate containing the same volume of medium as the assay plate.

3.3. Remove the assay plates from the incubator. Take off the breathable seal and r~~R~~e-seal the assay plate~~s~~ containing the compound-treated cells with a tight adhesive aluminum foil to ensure that no water will leak into the wells during the subsequent heating in the water bath. Ensure that the drilled holes in plate frame are accessible.

3.4. Place the assay plate and the ~~control~~dummy plate~~s~~ in the water bath with the bottom of the plate angled towards the water surface to force any remaining air out from under the plates.

3.5. Monitor the temperature inside the wells of the ~~control~~ dummy plate using a t~~T~~hermocouple ~~Traceable lab~~ thermometer.

3.6. Heat the assay ~~plates~~ plate ~~while floating~~ in the water bath for 3 minutes.

3.7. Immediately transfer the assay ~~plate~~ and ~~dummycontrol~~dummy plate to another water

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Commented [A3R2]: Bra kommentar, vi bör nog skriva att man bör kolla DMSO tolerabilitet för varje cell linje istf att skriva ut 0.1% som gräns. Som vi ju inte höll oss till som sagt...

bath with ~~room tempered~~room-tempered water to cool down for 5 minutes. The assay plate is now ready for further processing.

4. Fixation

4.1. Dispense 10 µL 16% (w/v) paraformaldehyde (PFA) ~~PFA~~ directly to the assay plate~~s~~ using a ~~Multidrop~~ bulk reagent dispenser or a multichannel pipet. Incubate at room temperature for 20 minutes. Note: some fixatives are classified as carcinogenic and institutional safety regulations should be followed.

4.2 Aspirate the PFA solution and wash the cells with 300 ~~µl~~ µL PBS ~~(overflow protocol)~~ using a plate washer. Place the plate on the plate washer and select the aspiration program. Note: This procedure has been optimized using an overflow protocol on the plate washer in which liquid is simultaneously dispensed and removed. If a plate washer or similar procedure is not available the washing step can alternatively be done manually.- *Manual washing procedure:* Remove the liquid from the wells by inverting the plate with a rapid hand twist over a waste tray or sink. Complete removal of liquid is essential for good performance. Add 80 µl PBS with a multi-channel pipette and invert the plate again as described ~~following the same procedure as~~ above, repeat the washing procedure two times. After the last wash blot the plate against clean paper towels to remove any excess liquid.

~~4.2. the plate can be washed with 3 x 50 µL PBS manually by turning the plate upside down and "flicking" out the liquid followed by addition of PBS. Dab the plate with a paper towel in between to remove excess liquid remaining on the plate.~~

5. Permeabilization

5.1. Add 20 µL 0.1% (v/v) NP-40 to the wells with a multichannel pipet and incubate at room temperature for 10 minutes. Wash the cells using the same procedure as described above (step 4.2).

5.2. Alternatively, when appropriate, apply an antigen retrieval protocol, e.g. add 80 µL 10 mM glycine at pH 7.2 and incubate for 10 minutes at room temperature. Aspirate the glycine solution using a plate washer by placing on the plate washer and selecting the aspiration protocol. See notes in 2.1 and 4.2 if a plate washer is not available. ~~a~~Add 20 µL 1% SDS to the wells with a multichannel pipet. Incubate at room temperature for 5 minutes and wash according to the same procedure described above (step 4.2).

6. Blocking

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6.1. Add 15 µL 1% (w/v) ~~BSA~~ bovine serum albumin (BSA) in PBS to the wells using a ~~Multidrop~~ bulk reagent dispenser or a multichannel pipet. Incubate the plate at room temperature for 1 hour or overnight at 4 °C with an aluminum foil plate seal.~~.~~

7. Primary antibody

7.1. Aspirate the blocking solution using a plate washer by placing on the plate washer and selecting the aspiration protocol. See notes in ~~2.1 and~~2.1 and 4.2 if a plate washer is not available.~~.~~

7.2. Add 10 µL primary antibody diluted accordingly in 1% (w/v) BSA in PBS to the wells using a multichannel pipet. ~~Incubate~~ Incubate the plate at room temperature for 1 hour or overnight at 4 °C with an aluminum foil plate seal.

8. Secondary antibody

8.1. Aspirate the primary antibody solution and wash the wells according to same procedure as described above (step 4.2).

8.2. Add 10 µL Alexa 488 secondary antibody diluted accordingly in 1% (w/v) BSA in PBS. Incubate at room temperature for 1 hour. Seal the plate with an adhesive aluminum foil seal to protect from light. Note: Protect the plate from light during this and subsequent steps.

9. Nuclear staining and ~~Cell~~ cell mask~~mask~~

9.1. Add 10 µL ~~Hoechst~~ nuclear dye diluted to 0.05 mg/mL in PBS to ~~all~~ the wells using a multichannel pipet. Incubate at room temperature for additional 10 minutes.

9.2. Aspirate the secondary antibody and Hoechst solution and wash the wells using the same procedure as described above (step 4.2).

9.3. Add 10 µL ~~HCS cell mask~~cell mask diluted to 200 ng/mL in PBS to the wells using a multichannel pipet. Incubate at room temperature for 30 minutes.

9.4. Aspirate the cell mask solution and wash the wells using the same procedure as described above (step 4.2).

9.5. Dispense 60 µL PBS to all wells using the plate washer a bulk reagent dispenser or a multichannel pipet and ~~re~~ seal the plates with an adhesive aluminum foil.

10. Image acquisition and analysis

10.1. Capture images on ~~ImageXpress Micro XLS Widefield High Content Analysis System (Molecular Devices)~~a high content imager using 3 fluorescent channels: DAPI (387/447), GFP

(472/520) and TexasRed (562/624).

10.2. Acquire 4 images per well using 10X ~~Pan-Fluor 0.3 NA~~ objective. Use automated laser autofocus and apply binning 2 during acquisition.

10.3. Store images as 16 bit, gray scale tiff files along with metadata.

10.4. Analyze images using available software~~using MetaXpress Custom Module~~. Identify cell boundaries using Cell Scoring algorithm with DAPI (nucleus) and TexasRed (cytoplasm).

10.5. Extract average intensity for all acquired wavelengths for further data analysis.

10.6. Calculate Z-factor to ensure the robustness of the assay.

~~**10.5.**~~ 10.7. Calculate % stabilization using the following formula: 100*(1-(well intensity – average well intensity negative control)/(average intensity positive control-average intensity negative control)). Since the maximum stabilization between compounds can vary and in fact be greater than the positive control for ITDRF curves the maximum and minimum stabilization intensities for each compound are sometimes used in place of the ~~control intensity values~~intensity values of the control wells.~~For ITDRF curves~~

REPRESENTATIVE RESULTS:

The protocol outlined in **Figure 1** describes the basic workflow for running CETSA assays on adherent cells with detection of remaining soluble protein by high content imaging. This workflow can be easily adapted to all stages of assay development by modifying the plate layout of the compounds or reagents[14]. We detail expected results for several anticipated use cases below.

Antibody identification ~~for~~ and assay development. A prerequisite for successful results is the identification of a primary antibody or other suitable affinity reagent that selectively recognizes the native form of the protein in the presence of the aggregated and precipitated protein formed during the heat challenge in step 3. To establish the CETSA imaging assay described here we screened a panel of 9 antibodies targeting p38α at 52°C for signal window between the positive and negative controls. We then titrated the best antibodies and settled on the conditions shown in **Figure 2 A, B** with~~shows~~ representative immunofluorescence images for p38α stabilized by a known ligand (positive control) and ~~vehicle~~ DMSO (negative control). Antibody recognition should also not be disrupted by the conformational changes of the target protein that may be induced by ligand binding (**Figure 2C**). As an example BIRB796 has a long off rate and quantification of target engagement was only possible by applying an antigen retrieval step 5.2 (**Figure 2D**). It is important to validate the performance of the primary antibody with known ligands covering different binding sites of the target protein if available. The antibody validation is preferably done both with and without the antigen~~-the~~ retrieval step 5.2.~~ As an example BIRB796 has a long off rate and quantification of target engagement was only possible by applying an antigen retrieval step 5.2 (**Figure 2D**).~~

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Commented [A7R6]: Nej, behövs inte tycker jag

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Commented [A8]: Is this right?

Ja det ska stämma, behöver vi på-peka att negative kontroll är vehicle och positive control är referens substans.

Bör vi lägga till i substans behandlingsdelen att kontroller ska inkluderas på varje platta?

T_{agg} and ITDRF curves. As mentioned above CETSA experiments can be run in two different modes, T_{agg} curves and ITDRF experiments. Both variants utilize the same basic protocol outlined in **Figure 1** and in the protocol section. In the first setup the purpose is to challenge the cells with a temperature gradient and compare the T_{agg} curves in the presence and absence of a single concentration of ligand. To perform a T_{agg} curve separate plates are heated for 3 minutes ~~at~~ across a range of temperatures. In performing this experiment it is important to time the compound treatment length for each plate with the time it takes for the water bath to stabilize to the new temperature. In this regard the peforming the heat challenge step ~~heating~~ in the water bath is more ~~challenging~~ time consuming than heating tubes in~~with~~ a PCR machine. The experimental path ~~second alternative~~ is to next run concentration response curves of a ligand at a fixed temperature ~~and~~ to generate ITDRF curves. In general when testing multiple compounds ~~we prepare~~ assay ready plates for the compound addition are prepared ~~of pre-diluted compounds~~using automated liquid handling to achieve the most reproducible data. Compounds are serially diluted in DMSO and then dissolved in cell culture media to the desired ~~stock~~ concentrations. We have typically tested 11 point concentration series starting at 50 – 100 µM in 3 or 4 fold dilution, but this depends on the potency of the ligands used. It is advised to first establish the T_{agg} curve both in absence and presence of a ligand and select the temperature for subsequent isothermal experiments where a shift between the curves can be observed. The selected temperature should be around or just above the T_{agg}. Both formats allow for confirmation of target engagement but for ranking of compound affinities ITDRF experiments are often more suitable. **Figure 3A** shows an illustration of anticipated quantified results for a Tagg curve and **Figure 3B** quantified results of a typical ITDRF experiment.

Screening campaign. The protocol can also be adapted to screening campaigns to identify novel binders of the target protein. In this case i~~I~~sothermal heat challenges ~~can also be~~are applied ~~in screening mode where~~for a large number of compounds ~~is tested~~ at a single concentration followed by ITDRF experiments for identified stabilizing compounds. We prepare assay ready screening plates and transfer the compounds ~~stocks~~diluted in culture media to the assay plates using automated liquid handling. As with all thermal stability assays it is necessary to exceed the dissociation constant to observe protein stabilization and thus we have applied small molecule libraries at 50 µM to facilitate hit identification. Triaging of the hits using ITDRF will later allow ranking and prioritization of these compounds. **Figure 4** shows a representative result~~s~~ from a screening plate.

~~*Troubleshooting.* Uneven heating of the plates can cause fluctuations and plate-to-plate variations in the observed data. Since the plates are partly submerged in a water bath during the transient heat challenge (step 3.4) the outer wells of the plates are exposed to the hot water not only at the bottom of the wells but also through the outer wall. This may cause higher temperature during the same submersion time in the outer wells of the plate and subsequent plate edge effects (illustrated in **Figure 5**) Therefore, it is recommended to avoid the outer wells of the plate. Similar effects can also be seen if air bubbles are trapped under the plate during the heating step preventing sufficient contact between the hot water and the bottom of the wells. We have also explored the use of several cell lines, and have found~~

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Commented [A9]: Jag fick inte till det så bra, men jag tycker att det är värdefullt att hora tag +/- substans och leta efter ett stort assay fönster vid typ så låg temp som möjligt, eller hur?

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~~variability surface attachment after the heat challenge. Preliminary experiments in our labs suggest that the use of a coating or extracellular matrix such as Synthemax II-SC or Cell-Tak can minimize cell detachment.~~

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic overview of the protocol described in this article.

Figure 2 . A) Example data for detection of human p38α in A-431 cells. Representative images of positive (1 µM AMG548~~ 1µM~~) and negative (DMSO) controls. Red- nuclear Hoechst staining, Green- p38α staining. Images taken with 10X magnification and the white scale bar represents 73 µm.~~.~~ B) Example of quantified data expressed as average intensity/cell. Error bars represent standard error of mean of 16 replicates for ~~5~~ 6 separate plates. Each plate was heated to 52°C in a water bath followed by fixation of the cells, permeabilization and subsequent immunostaining. C) ~~Immunfluorescence~~Immunofluorescence signal intensity measured after treating ~~A431~~A-431 cells with 1 µM BIRB796 or DMSO as~~e~~ described above followed directly by fixation. In the absence of a heating step the BIRB796 signal is lower than the DMSO signal, suggesting that BIRB796 disrupts the detection of p38α with this antibody. D) $ITDRF_{CETSA}$ curves for cells treated with BIRB796 either with (▲) or without (▲) antigen retrieval protocol in step 5.2. This figure has been modified from [14]. Copyright 2018 American Chemical Society.
~~Figure adapted from [14]. Copyright 2018 American Chemical society.~~

Figure 3 A) Example data for detection of human p38α in A-431 cells. A) Thermal aggregation curve experiments for cells treated with positive~~,~~ (1 µM AMG548~~ 1µM~~, in orange)~~,~~ and negative (DMSO~~,~~ in grey)~~,~~ controls. Error bars represent standard error of mean of 32 ~~to~~ or 464 replicates. B) $ITDRF_{CETSA}$ experiment performed at 52°C for cells treated with serial dilutions of SB203580 in blue, Skepinone-L in green and RWJ67657 in red. Error bars represent standard error of mean of 6 replicates. Quantified data are expressed as average intensity/cell and normalized against the highest concentration of respective compound. This figure has been modified from ~~Figure adapted from~~ [14]. Copyright 2018 American Chemical ~~society~~Society.

Figure 4. ~~Respresentative~~Representative overview of a screening plate at 10X magnification. Positive controls (1 µM AMG548) are in columns 2 and 24 and negative controls (DMSO) are in columns 1 and 23. All other wells contain 50 µM of the library compounds used for screening with several hits denoted. In the inset figures the white line in the lower right corner represents 200 µm. This figure has been modified from [14]. Copyright 2018 American Chemical Society.

~~Figure adapted from [14]. Copyright 2018 American Chemical society.~~

~~Figure 5. Heatmap depicting the representative signal intensity across an entire a plate with visible edge effects. Columns 1 to 24 were treated with DMSO (white to red in the heatmap) and middle wells treated with 1 µM AMG 548 were plated in rows D, G, J, M from columns 5-20 (grey in the heatmap). The plate was heated to 52°C on a flat block PCR adapter. Figure~~

~~adapted from [14]. Copyright 2018 American Chemical society.~~

DISCUSSION:

As discussed in the results section there are several key steps to the procedure. First it is important to identify a high quality affinity reagent. We recommend screening a small library of antibodies for each desired target. After a primary antibody has been selected it is also important to validate the system for a number of different binding sites of the protein target if appropriate. Counter~~-~~screening for compounds that interfere with the assay signal as shown in Figure 2C by omitting the heat challenge is highly encouraged after a screening campaign. When a reduced signal in presence of a ligand is observed an ~~, this occurs~~ antigen retrieval protocol~~s~~ as described in step 5.2 can be tested. Uneven heating of the plates can cause fluctuations and plate-to-plate variations in the observed data. Since the plates are partly submerged in a water bath during the transient heat challenge (step 3.4) the outer wells of the plates are exposed to the hot water not only at the bottom of the wells but also through the outer wall. This may cause higher temperature during the same submersion time in the outer wells of the plate and subsequent plate edge effects ~~(illustrated in **Figure 5**)~~. Therefore, it is recommended to avoid the outer wells of the plate. Similar effects can also be seen if air bubbles are trapped under the plate during the heating step preventing sufficient contact between the hot water and the bottom of the wells. Given the challenges with heating imaging plates, ~~It is important to heat the plate evenly, and d~~devices and plates specifically made ~~to heat imaging plates~~for exposure to heat could help advance this method. We have ~~also~~ explored the use of several cell lines, and have found variability in surface attachment after the heat challenge for adherent cell types. Preliminary experiments in our lab~~s~~ suggest that the use of a coating or extracellular matrix such as Synthemax II-SC or Cell-Tak can minimize cell detachment, but this could pose a ~~problem~~technical challenge when usin~~g~~ ~~fog~~r semi-attached cell types. Since compound treatment is performed in live cells, the small molecules must be cell permeable. If the compound is not membrane permeable, alternative high throughput CETSA methods are recommended. Some compounds need to be metabolically activated to bind to their target proteins[11]. In such cases longer treatment cells with the compound prior to the heat challenge is recommended.

~~Finally if detachment of cells is observed during the heat challenge, the use of matrixes such as Synthemax II or Cell-Tak have given promising preliminary results to maintain cell adherence.~~

This protocol requires a single plate from cell seeding to imaging making it amenable to high throughput screening campaigns. The number~~s~~ of cells for each experiment is much lower than can be achieved with western blots or previous AlphaScreen assays[15]. Furthermore washing or cell detachment steps, which may alter compound availability and ~~binding~~binding, are removed, preserving the established binding equilibrium through the heat challenge step. Unlike previous procedures, a lack of signal due to cytotoxicity is simultaneously reported on by nuclear staining. Finally, imaging preserves spatial resolution of the individual cells allowing for target engagement measurements in mixed populations of cells or cell states.

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Troubleshooting. Uneven heating of the plates can cause fluctuations and plate-to-plate variations in the observed data. Since the plates are partly submerged in a water bath during the transient heat challenge (step 3.4) the outer wells of the plates are exposed to the hot water not only at the bottom of the wells but also through the outer wall. This may cause higher temperature during the same submersion time in the outer wells of the plate and subsequent plate edge effects (illustrated in **Figure 5**) Therefore, it is recommended to avoid the outer wells of the plate. Similar effects can also be seen if air bubbles are trapped under the plate during the heating step preventing sufficient contact between the hot water and the bottom of the wells. We have also explored the use of several cell lines, and have found variability surface attachment after the heat challenge. Preliminary experiments in our labs suggest that the use of a coating or extracellular matrix such as Synthemax II-SC or Cell-Tak can minimize cell detachment.

To truly assess the applicability of the approach concerted efforts to apply the technique across the melting proteome are needed. Such experiments will clarify theThe ease of identifying suitable affinity reagents, which report selectively on the native protein against a background ofin the presence of the remaining denatured and aggregated proteins is unknown. We currently do not know how protein expression levelsthe abundance of the target of interest will affect the ability to reliably measure a signal window., although Wwe expect this to reflect correlate strongly to antibody affinity and selectivity of the available antibodies, nor how variation in abundance across cell lines will affect assay optimizationsand anticipate that for low abundant proteins that technologies used to enhance that enhance signal in immunofluorescent assays to bewill be applicable. Alternatively, tagged proteins or functionalized compounds could be used for detection in modified versions of the described protocol. This protocol is limited to targets that melt and for interactions where a quantifiable stabilization can be observed. For example, endogenous ligands can alter protein meltingstabilize a protein in live cell assays, which may prevent further stabilization by an exogenous ligand. Since compound treatment is performed in live cells, the small molecules must be cell permeable. While this may be a benefit for some screening applications, if the compound is not membrane permeable, alternative high throughput CETSA methods are recommended. Although not explored in the described protocolhere, we believe that it will be possible to multiplex readouts to allow for the study of multiple targets simultaneously, or a target with downstream effectors. Further studies are necessary to investigate these aspects of *in situ* CETSA.

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DISCLOSURES:

The authors have no disclosures to report.

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