Journal of Visualized Experiments

An automated differential nuclear staining assay for accurate determination of mitocan cytotoxicity

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61295R1
Full Title:	An automated differential nuclear staining assay for accurate determination of mitocan cytotoxicity
Section/Category:	JoVE Cancer Research
Keywords:	high-throughput screening; mitochondria-targeting drugs; cytotoxicity assay; leukemia; fluorescence microscopy; automated cell counting.
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Houston, TX, USA

TITLE:

An Automated Differential Nuclear Staining Assay for Accurate Determination of Mitocan Cytotoxicity

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KEYWORDS:

High-throughput screening, mitochondria-targeted compounds (mitocans), cytotoxicity assay, leukemia, fluorescence microscopy, automated cell counting.

SUMMARY:

The protocol describes a rapid, high-throughput, reliable, inexpensive, and unbiased assay for efficiently determining cellular viability. This assay is particularly useful when cell mitochondria have been damaged, which interferes with other assays. The assay uses automated counting of cells stained with two nuclear dyes – Hoechst 33342 and propidium iodide.

ABSTRACT:

The contribution of mitochondria to oncogenic transformation is a subject of wide interest and active study. As the field of cancer metabolism becomes more complex, the goal of targeting mitochondria using various compounds that inflict mitochondrial damage (so-called mitocans) is becoming quite popular. Unfortunately, many existing cytotoxicity assays, such as those based on tetrazolium salts or resazurin require functional mitochondrial enzymes for their performance. The damage inflicted by compounds that target mitochondria often compromises the accuracy of these assays. Here, we describe a modified protocol based on differential staining with two fluorescent dyes, one of which is cell-permeant (Hoechst 33342) and the other of which is not (propidium iodide). The difference in staining allows living and dead cells to be discriminated. The assay is amenable to automated microscopy and image analysis, which increases throughput and reduces bias. This also allows the assay to be used in high-throughput fashion using 96-well plates, making it a viable option for drug discovery efforts, particularly when the drugs in question have some level of mitotoxicity. Importantly, results obtained by Hoechst/PI staining assay show increased consistency, both with Trypan blue exclusion results and between biological replicates when the assay is compared to other methods.

INTRODUCTION:

The first step to identifying effective cancer treatments is the selection of a robust, unbiased cytotoxicity assay that can be used to examine the effect of treatment. A common choice for low-throughput experiments is the exclusion of Trypan blue dye from living cells. This method is favored because it allows a relatively unbiased method for quantifying cell survival. Trypan blue passively diffuses into cells whose membranes are compromised, but it is effectively blocked from entering healthy cells[1]. The quotient of the living cells and the total cells represents the percent viability, which indicates the efficacy of the treatment. The most significant disadvantage of the Trypan blue assay is that it is poorly suited for high-throughput methodologies. It has a relatively low signal-to-noise ratio and prolonged staining can result in artifacts due to the staining of viable cells. Consequently, Trypan blue exclusion is typically, but not always[2], relegated to manual counting. This makes it too slow and introduces a strong possibility of bias due to subjective judgment of the researcher (unless blinding or independent counts are used, which further reduce laboratory throughput). In general, the throughput of this assay is insufficient for modern drug discovery.

Viability assays, which generally have a much higher throughput, allow researchers to circumvent this limitation, but come with significant caveats (**Table 1**). These methods generally fall into two groups. One group is comprised of colorimetric assays that are based on the function of cellular redox enzymes. Colorless or non-fluorescent substrates are converted into vibrant products that can be quantified using a spectrophotometer. Classic examples include tetrazolium salts (MTT, WST-1, XTT, etc.) and resazurin. This category also includes luminescent assays that utilize luciferin to evaluate ATP level. Assays of this type have the underlying limitation that they are measuring cellular metabolism, which is not cellular viability *per se.* It is quite common for cells to become quiescent under adverse conditions, but still retain the ability to divide[3-5]. For example, cancer stem cells are often relatively metabolically quiescent[6-9], and are likely to be difficult to assay using these techniques. Effectiveness of treatments that damage mitochondrial function, such as most mitocans, is also likely to be significantly overestimated.

An alternative methodology leverages the chemical properties of various substances that allow them to either cross or not cross biological membranes[10-18]. One example is nuclear stains such as SYTOX or propidium iodide (PI). This category also includes assays that are similar in concept but different in function, such as the lactate dehydrogenase (LDH) assay, which measures the release of LDH into the extracellular milieu as an indicator of cellular necrosis (**Figure 1, Table 1**). These assays are more capable of distinguishing between metabolically inactive and dead cells.

Recent studies have demonstrated that mitochondrial metabolism is altered in some cancers[19-25]. For example, acute myeloid leukemias have been shown to upregulate their mitochondrial mass, mtDNA content, and mitochondrial respiration to meet their energy demands[19,26,27]. On the other hand, some solid tumors are characterized by mitochondrial dysfunction, or rather "metabolic reprogramming", such as downregulation of mitochondrial proteins involved in

OXPHOS or decreased mtDNA content, that has been associated with tumor invasiveness, metastatic potential and resistance to apoptosis-inducing drugs[28,29]. Furthermore, recently, there has been an increased interest in using mechanistically diverse compounds that affect mitochondrial function (generally called mitocans[30]), as potential therapies for particular cancers. These drugs target ATP synthesis, mitochondrial DNA, OXPHOS, and ROS production, as well as pro- and anti-apoptotic proteins associated with mitochondria[30,31]. Several studies have shown that this approach has significant promise[19,32-34]. However, these metabolic deviations in cancer cell biology or mitochondria-targeting treatments may significantly affect conventional viability assays that are based on mitochondrial functionality.

Here, an optimized protocol for a differential nuclear staining assay is described. The protocol allows fast and accurate determination of cytotoxicity of mitocans or their combinations with other compounds. Hoechst 33342 is a cell-permeant nuclear dye that readily crosses cell membranes to stain DNA, allowing the total cell count to be obtained. By co-staining with PI, which only enters the nuclei of dead cells, the proportion of living (Hoechst only) and dead (stained with both) cells can be accurately determined. This protocol refines the published assay[35] by adding a step for the optimization of the dye concentration (by cross-referencing results with orthogonal Trypan blue method) and centrifugation of the plate prior to imaging. Since many cell lines are semi-adherent or suspended, centrifugation increases the proportion of cells that are imaged and strongly improves accuracy. The assay has several advantages, including that staining does not require removal of media or washing. The dye mixture is also inexpensive, easy to prepare, and compatible with multichannel/robotic pipetting systems.

After cells have been stained, they are imaged with an automated microscope. This has the added advantage of creating a permanent record of the images that can be re-analyzed later and the effects of particular compounds can be re-evaluated by visual inspection of captured images. Once images have been obtained, cells can be counted either manually or by using any of several software packages, including both free (e.g., ImageJ, CellProfiler, etc) and commercial software (e.g., Metamorph, Gen5, etc.). Automated cell counting is generally preferable since properly developed automated cell counting pipelines are more accurate and less biased than manual counts. They also more effectively disregard cell debris or insoluble complexes. Development of these pipelines is generally straightforward and is simplified by the efficiency of the stains used. The output is quantitative since the actual number of dead cells is automatically calculated with respect to the total cell number, and different thresholds can be applied to increase or decrease the stringency of detection[35]. For convenience, optimized parameters for counting cells using software compatible with a commercial cell imaging multi-mode reader (e.g., Cytation 5) are included.

PROTOCOL:

1. Cytotoxicity assay: setup

1.1. Prepare solutions of compounds of interest at desired concentrations in the appropriate media (serum-free or 1, 2.5, or 5% FBS RPMI-1640).

1.1.1. To measure cytotoxicity of a single compound (e.g., to determine effective doses), prepare compounds at 2x final concentration.

1.1.2. To measure cytotoxicity of compound combinations, prepare compounds at 4x final concentration.

1.1.3. Prepare solvent-only controls by mixing the same amount of solvent with the appropriate medium. For example, if testing compounds dissolved in DMSO and methanol, make solvent-only control for each solvent.

1.2. Collect cells from culture dish or flask into a 15 mL conical tube.

1.3. Transfer 10 µL of cell suspension into a microcentrifuge tube and stain with 10 µL of 0.4% Trypan blue. Use a hemocytometer to count viable and non-viable cells for each cell source.

1.4. Pellet cells at 200 x *g* for 5 min. Aspirate or decant supernatant.

1.5. Resuspend cell pellet in assay-appropriate media (serum-free or 1, 2.5, 5% FBS RPMI-1640) at a cell density of 3 x 10^5 cells/mL.

NOTE: Cell density of 3 x 10^5 cells/mL provides a seeding density of 15,000 cells/well. Seeding density is an important parameter and ideally should be pre-defined prior to the experiment. Seeding density should take into account 1) cell size – usually larger cells are seeded at a lower density; 2) treatment duration – cells are typically seeded at a lower density for experiments that will last longer; and 3) cell division rate – cells with a higher rate of division are seeded at a lower density. Specific examples of optimized seeding densities: K562 cells, larger, 24 h duration – 10,000 cells/well; MOLM-13 cells, moderate size, 24 h treatment – 15,000/well; MOLM-13 cells, 48 h treatment – 8,000/well; small healthy peripheral blood mononuclear cells (PBMCs), 24 h treatment – 50,000/well; primary AML cells, 24 h treatment – 15,000-20,000/well (depending on availability). The presence of FBS in media may affect the activity of the compounds. Reducing the concentration of FBS may make assay results simpler to interpret, but it also reduces the physiological accuracy.

1.6. Seed 50 µL of cell suspension from step 1.5 into each well of a 96-well plate using a multichannel pipette.

1.7. Add compounds as follows:

1.7.1. For single compound assays, add 50 µL of 2x compound solution into each well. For solvent-control wells, add 50 µL of test media containing the solvent at the 2x concentration.

1.7.2. For combination assays, add 25 µL of each of the compound (4x solutions) into each well. For single compound-control wells, add 25 µL of 4x compound solution and 25 µL of test medium. For solvent-control wells, add 50 µL of test medium or test medium containing the solvent.

NOTE: The final concentration of DMSO should not exceed 0.5%. Add the media containing the compounds with the pipette touching the wall of each well due to low volume.

1.8. Gently tap the plate to ensure mixing of the contents of the wells.

1.9. Incubate plates at 37 °C in a humidified 5% CO_2 atmosphere for an appropriate time (e.g., 24 h).

2. Cytotoxicity Assay: staining with Hoechst 33342 and propidium iodide

2.1 Prepare 10x staining solution. Prepare this solution fresh before each experiment as it cannot be stored. Determine final dye concentrations prior to the experiment.

2.1.1. For leukemia cell lines and primary leukemia cells, use 1 mL of 10x staining buffer with 10 µL of 20 mM Hoechst 33342 and 50 µL of 1 mg/mL propidium iodide in sterile PBS (final concentrations: Hoechst 33342 20 µM, PI 5 µg/mL).

2.1.2. For healthy PBMCs, use 1 mL of 10x staining buffer with 10 µL of 20 mM Hoechst 33342 and 10 µL of 1 mg/mL propidium iodide in sterile PBS (final concentrations: Hoechst 33342 20 µM, PI 1 µg/mL).

NOTE: The final concentration of propidium iodide has to be determined prior to the experiments. Cells should be tested using a range of PI concentrations (1, 2.5, 5 µg/mL), and then Hoechst/PI-calculated viability should be compared with viability measured via Trypan blue. The PI concentrations listed above were chosen based on target cell viability in media-control wells (above ~90% for leukemia cell lines, above ~70% for primary normal PBMCs).

CAUTION: Hoechst 33342 and propidium iodide are potential carcinogens. Wear appropriate personal protective equipment when handling them.

2.2. After incubation, use a multichannel pipette to add 10 µL of 10x staining buffer to each well.

NOTE: To prevent cross-contamination, make sure that the pipette tips do not touch the media.

2.3. Gently tap the plate to mix and clear bubbles. Stain at 37 °C for 15 min.

2.4. Centrifuge the plate at 200 x *g* for 4 min to bring all the cells to the bottom of the plate. Carefully wipe the bottom of the plate with a damp laboratory wipe to remove fibers and/or debris that will interfere with imaging.

NOTE: Centrifugation of the plate ensures the highest chances for all the cells to be captured in the image. Often dead cells will detach and float, providing misleading values of cytotoxicity. Centrifugation mitigates this effect.

2.4.1. Image the plate as quickly as possible after centrifugation, ideally, within 15 min. Centrifugation can reduce the selectivity of PI staining and may allow cells to slowly accrue propidium iodide. The improvement in accuracy gained by visualizing the dead cells outweighs the slight increase in PI staining. Finish imaging within 1 h of centrifugation.

3. Cytotoxicity assay: data acquisition

3.1. Set the software for the automated microscope/plate imager to detect fluorescence for both Hoechst 33342 (excitation maximum 350 nm, emission maximum 461 nm) and PI (excitation maximum 493 nm, emission maximum 636 nm). Acquire images for each well in both channels.

3.2. Using the software (such as CellProfiler, a free image analysis studio based on MatLab http://cellprofiler.org/, ImageJ, or proprietary software such as Gen5) count the cells in each well in each channel.

NOTE: Since every cell should be stained with Hoechst 33342, and dead cells are stained with PI, the ratio of dead to all represents the fraction of cells that are dead. For example, if the automated count in untreated sample shows 467 cells stained with PI (dead cells) and 2335 cells stained with Hoechst 33342 (total cells), the fraction dead is 0.2 or 20%. This value is then compared to an identically handled sample where treatment was used.

3.3. Detailed description of the Hoechst/PI staining assay

3.3.1. Set the multimode plate reader/imager (e.g., Cytation5) to image cells in flat bottom, 96-well, generic black plastic plates.

3.3.1.1. Set an imaging protocol to utilize the standard DAPI and Texas Red filter sets. Take images at the well center using a 4x magnification objective. Use no offset (X/Y or Z).

3.3.1.2. Use the following settings used for DAPI filter set: LED - 10, integration time – 99, gain - 0; for Texas Red filter set: LED - 8, integration time – 950, gain - 18. Perform autofocusing based on DAPI signal; there was no offset in focusing between channels.

3.3.2. Perform image analysis using software (e.g., Gen5 v3.00).

3.3.2.1. In the software settings, define cells as shapes between 5 and 25 µm in their size. Exclude primary edge objects and split touching objects by turning on special option **Split touching objects**.

3.3.2.2. Process the image to remove the background (dark background subtraction), apply a nuclear mask (threshold value DAPI >= 6000 AU), and count objects. Perform a subpopulation analysis based on PI staining (threshold value Texas Red >= 5000 AU), and count objects.

3.3.2.3. Define cell viability %, as $(1 - \frac{Texas\ Red\ (counts)}{DAPI\ (counts)}) \times 100$.

REPRESENTATIVE RESULTS:

The aforementioned protocol has been developed using OCI-AML2 cells, which were taken as a representative acute myeloid leukemia (AML) cell line. AML is characterized by abnormal proliferation of undifferentiated and non-functional hematopoietic cells in the bone marrow[26]. Despite recent developments in AML targeted therapy, the standard of care has remained unchanged for several decades, and consists of induction therapy (typically comprised of three days of anthracycline [e.g., daunorubicin, idarubicin, or anthracenedione mitoxanthrone] and 7 days of cytarabine) followed by consolidation (typically comprised of rounds of cytarabine treatment followed by periods of recovery)[36].

Following the protocol outlined in **Figure 1A**, OCI-AML2 cells were seeded in 96-well plates and treated with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) at a range of concentrations. Cells were treated for 24 h at 37 °C in serum-free RPMI-1640 media, and then viability was assessed using Trypan blue (TB) exclusion or one of four viability assays (Hoechst/PI differential nuclear staining, LDH release assay, MTT, or alamarBlue). Representative images of cells stained with Hoechst/PI are shown in **Figure 2A**. Several important observations can be made immediately. First, the total number of cells (Hoechst staining) is reasonably high and is markedly greater than the number of dead cells (PI staining). This suggests that the media conditions are not triggering high rates of cell death. Second, since only cells being labeled with both Hoechst and PI are counted as dead (purple cells in the merged image), the probability of counting debris is very low. This image shows a good example of properly stained cells.

[Figure 1 and 2 here]

As we recently reported[19], leukemias are very sensitive to mitotoxic treatments, which indicates that the cells already have underlying mitochondrial damage. On this basis, we predicted that the MTT and alamarBlue assays, which are based on mitochondrial enzyme activity, would inaccurately measure cellular viability. As expected, these assays (especially alamarBlue) showed significantly lower viability compared to Trypan Blue exclusion, see **Figure 2B,C, Supplementary Tables S1,S2**). This is consistent with the fact that doses of these

compounds required to induce apoptosis or necrosis are higher than those needed to compromise mitochondrial function.

Amongst the assays that were tested, dual staining with Hoechst 33342 and PI had the best combination of robustness, sensitivity, and consistency with TB staining, with the smallest median deviation from TB exclusion method after CCCP or 2-DG treatment (**Supplementary Tables S1-2**). Interestingly, at higher doses of CCCP (50 and 100 µM) viability estimated with Hoechst/PI or TB was increased compared to lower doses (**Figure 2B,** top). This is likely due to the precipitation of CCCP at higher doses due to its hydrophobicity, reducing its effective concentration and impact on cells. Increasing the dose of CCCP further reduced Hoechst/PI-estimated mean viability of OCI-AML2 cells, however: 74% at 150 µM, 62% at 200 µM, 6% at 300 µM (*data not shown*).

The Hoechst/PI assay was also effective at determining cellular viability after treatment with other mitochondria-targeting molecules. These included rotenone, a poison that compromises Complex I of the mitochondrial electron transport chain[37], and 3-bromopyruvate, a glycolytic inhibitor and alkylating agent that impairs mitochondrial respiration and mitochondrial metabolism[38] (**Figure 3A-D, Supplementary Tables S3-4**).

[Figure 3 here]

The Hoechst/PI cytotoxicity assay was further validated using a panel of leukemia cell lines representing multiple types of leukemia, as well as primary cells. They included MOLM-13 (an AML cell line), primary AML cells derived from a representative patient sample, MOLT-4 (an acute lymphoblastic leukemia cell line, ALL), and K562 (a chronic myelogenous leukemia cell line, CML) (**Figure 3E, Supplementary Figure S1**). Results of the Hoechst/PI assay showed that these cells had profound differences in rotenone sensitivity, ranging from very sensitive (MOLT-4) to resistant (K562) cells.

To demonstrate the robustness of the assay, OCI-AML2 cells were treated with a concentration gradient of 3-bromopyruvate, as described in the protocol above. Cell counts were collected for 6 wells at each concentration and are shown (**Figure 4A,B**). These counts were used to calculate viability and showed that as few as 4 wells were sufficient to accurately capture assay outcome (**Figure 4C**). The resulting dose-response curve is shown (**Figure 4D**).

[Figure 4 here]

Although the assay is relatively robust, care must still be taken. Improper performance of the assay may compromise its accuracy. Several compromised outcomes are shown for troubleshooting purposes (**Figure 5**). The first set of images shows the consequences of over-staining with PI, which may arise from the following sources: using the dye at too high concentration, staining for too long, or using too high LED intensity/integration time in the red channel (**Figure 5A**). These errors will generate artificially inflated numbers of dead cells. The second issue arises from neglecting the centrifugation step. Often, dead cells begin to lose their

attachment from the surface of the dish. Consequently, they will be underrepresented during image acquisition, which generally occurs near the bottom of the well (**Figure 5B**). Finally, overexposure in the Hoechst channel artificially expands the size of the cells, significantly reducing the total counts per well and limiting the assay power (**Figure 5C**).

[Figure 5 here]

FIGURE AND TABLE LEGENDS:

Table 1. List of cytotoxicity assays. Cytotoxicity assays, some of which were used in this study, listed along with the brief description of their key features.

Figure 1. Experimental timeline and comparison of existing cytotoxicity assays. (**A**) Flowchart summarizing the timeline for the experimental procedure, e.g. Hoechst/PI staining. (**B**) Comparison of cytotoxicity assays, some of which were used in this study. Dye exclusion assays involve impermeant nuclear dyes that stain dead cells with compromised plasma membrane: TB – Trypan blue, PI – propidium iodide, EtBr – ethidium bromide, and SYTOX. Second group of assays depend on cellular metabolism, for example, tetrazolium salts MTT, XTT, and CKK-8 (WST-8), resazurin-based reagent alamarBlue, etc.

Figure 2. Comparison of a panel of cytotoxicity assays with Trypan blue exclusion. (**A**) Representative images of total (Hoechst 33342) and dead (propidium iodide, PI) OCI-AML2 cells via Hoechst/PI staining. (**B**) Assessment of viability of OCI-AML2 cells using different methods after treatment with a gradient of CCCP (**top**) or 2-DG (**bottom**) concentrations. OCI-AML2 were treated with CCCP or 2-DG in serum-free RPMI-1640 for 24 h prior to determination of cell viability. Shown is mean, error bars represent SEM. **C.** Difference in cell viability between cytotoxicity assays in (**B**) *vs.* Trypan blue staining (see **Supplementary Tables S1-2** for the exact numbers and median difference). Stars indicate significant difference *vs*. Trypan blue staining. ** $p < 0.01$, *** $p < 0.001$, ns – non-significant. Group comparison was done via *t*-test with correction for multiple hypothesis testing. Three independent biological replicates were performed.

Figure 3. Validation of Hoechst/PI cytotoxicity assay in leukemia cells. (**A,B**) OCI-AML2 (AML) cells were treated with different concentrations of rotenone (**A**) or 3-bromopyruvate, 3-BP (**B**) in serum-free RPMI-1640 media for 24 h, then viability was determined. Shown is mean with SEM. (**C,D**) Comparison of viability difference between Hoechst/PI assay *vs.* Trypan blue staining (for the cells in **A-B**, see **Supplementary Tables S3-4** for quantitation). Stars indicate statistical significance *vs.* Trypan blue staining. ** $p < 0.01$, *** $p < 0.001$, ns – non-significant. Group comparison was done via *t*-test with correction for multiple hypothesis testing. **E**. MOLM-13 (AML), primary AML cells isolated from a patient, MOLT-4 (ALL), and K562 (CML) cells were treated with the indicated concentrations of rotenone in serum-free RPMI-1640 media for 24 h, prior to viability determination using Hoechst/PI staining. Shown is mean with SEM. Three independent biological replicates were performed.

Figure 4. Reproducibility of Hoechst/PI staining. Viability of OCI-AML2 cells after 24 h of treatment with different concentrations of 3-bromopyruvate (3-BP). (**A**) Total cell number counted via Hoechst 33342 staining, (**B**) Dead cell number counted via propidium iodide staining. (**C**) Viability (%) calculated using (**A-B**). (**D**) Representative dose-response graph. Shown is mean with SEM. Three independent biological replicates were performed.

Figure 5. Comparison of optimal and sub-optimal assay parameters. Comparison of results acquired via optimized parameters (left) or sub-optimal parameters (right). (**A**) Normal PBMCs were stained with propidium iodide at 1 µg/mL (left) or 5 µg/mL (right) for 15 min before imaging. (**B**) Images of OCI-AML2 cells taken with (left) or without (right) plate centrifugation. (**C**) Images of OCI-AML2 cells acquired with optimal (left) or excessive (right) integration time for the Hoechst channel.

Supplementary Figure S1. Representative images of cells stained with Hoechst/PI, with or without rotenone treatment at indicated concentrations in serum-free RPMI-1640 media for 24 h: A-B. AML: MOLM-13 cell line (**A**), and primary AML cells (**B**) derived from a representative patient sample. **C.** ALL: MOLT-4 cell line. **D.** CML: K562 cell line.

Supplementary Table S1. Viability differences for a panel of assays in OCI-AML2 cells after CCCP treatment. Assessment of viability was performed after 24 h of treatment with different concentrations of CCCP. Median viability differences between tested assays (Hoechst/PI, LDH, MTT, or alamarBlue) and Trypan blue staining with automated counting was calculated based on viability differences at each drug concentration.

Supplementary Table S2. Viability differences for a panel of assays in OCI-AML2 cells after 2-DG treatment. Assessment of viability was performed after 24 h of treatment with different concentrations of 2-DG. Median viability differences between tested assays (Hoechst/PI, LDH, MTT, or alamarBlue) and Trypan blue staining with automated counting were calculated based on viability differences at each drug concentration.

Supplementary Table S3. Viability difference between Hoechst/PI and TB exclusion method in OCI-AML2 cells after rotenone treatment. Assessment of viability was performed after 24 h of treatment with different concentrations of rotenone. Median viability difference between Hoechst/PI and Trypan blue staining with automated counting was calculated based on viability differences at each drug concentration.

Supplementary Table S4. Viability difference between Hoechst/PI and TB exclusion method in OCI-AML2 cells after 3-bromopyruvate (3-BP) treatment. Assessment of viability was performed after 24 h of treatment with different concentrations of 3-BP. Median viability difference between Hoechst/PI and Trypan blue staining with automated counting was calculated based on viability differences at each drug concentration.

DISCUSSION:

Although the protocol for Hoechst/PI cytotoxicity assay is robust and requires comparatively

little hands-on time, there are several experimental details that are very important to ensure accurate results. First, it is essential to make sure that the concentration of DMSO remains below 0.5% (v/v). It is generally agreed that exposure to even low doses of DMSO can substantially alter the morphology and attachment of cells and significantly delay cell cycle progression[39,40].

Second, the staining should be performed as soon as possible after treatment. Since there are no washing steps, the compound remains in the wells and can continue to inflict damage on the cells. In comparison to the earlier protocol[35], the cells were stained for only 15 minutes immediately after treatment. This limits the possibility that viable cells could be inadvertently stained with propidium iodide. For this reason, we suggest staggering treatments and staining if more than two plates will be treated. This allows for imaging time between plates and improves reproducibility.

Third, appropriate dye concentration is dependent on cell type. It is important to empirically determine the optimized propidium iodide concentration, beginning with untreated cells, using Trypan blue staining as an orthogonal reference.

Finally, the centrifugation step immediately before visualization is also critical. Based on this protocol, a range of 500-4,000 cells is recorded (depending on seeding density). This is a notable improvement over the 100-400 cells per well used previously[35]. Considering the variation in the population of cancer cells (especially primary cells), having more cells for analysis is important, and may allow more robust data analysis.

Due to the relative simplicity of the method, a wide variety of modifications can easily be performed. For example, many other cell-impermeant dyes are available from a number of vendors and can be substituted for propidium iodide. While this substitution has relatively little value on its own, the increased palette of colors means that more complex experiments can be performed. For example, the addition of a third color will allow differential survival to be assessed between subpopulations of cells, as is commonly performed with flow cytometry.

A more substantial modification of the protocol is foregoing cell visualization and using a spectrophotometer instead. This approach has the advantage of utilizing a nearly ubiquitous piece of equipment (a standard spectrophotometer with a microplate reader) and is the fastest method to acquire data. However, this method is far less accurate. Fluorescence intensity is representative of cell staining, but stochastic variations in staining intensity, combined with the relatively smaller sample area, introduce significant variability. While further optimization (such as the inclusion of a fixative, additional washing steps, or a larger number of samples) may address these problems, fluorescence microscopy is generally a superior approach.

In conclusion, the modified Hoechst/PI protocol is a fast, accurate, inexpensive, high-throughput cytotoxicity assay that is independent of mitochondrial function. This assay has substantial utility for efficiently screening compounds or compound combinations that target mitochondria.

ACKNOWLEDGMENTS:

NVK, a CPRIT scholar in Cancer Research, thanks the Cancer Prevention and Research Institute of Texas (CPRIT) for their generous support, CPRIT grant RR150044. This work was also supported by the Welch Foundation Research Grant C-1930, and by the National Institutes of Health R35 GM129294 awarded to NVK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

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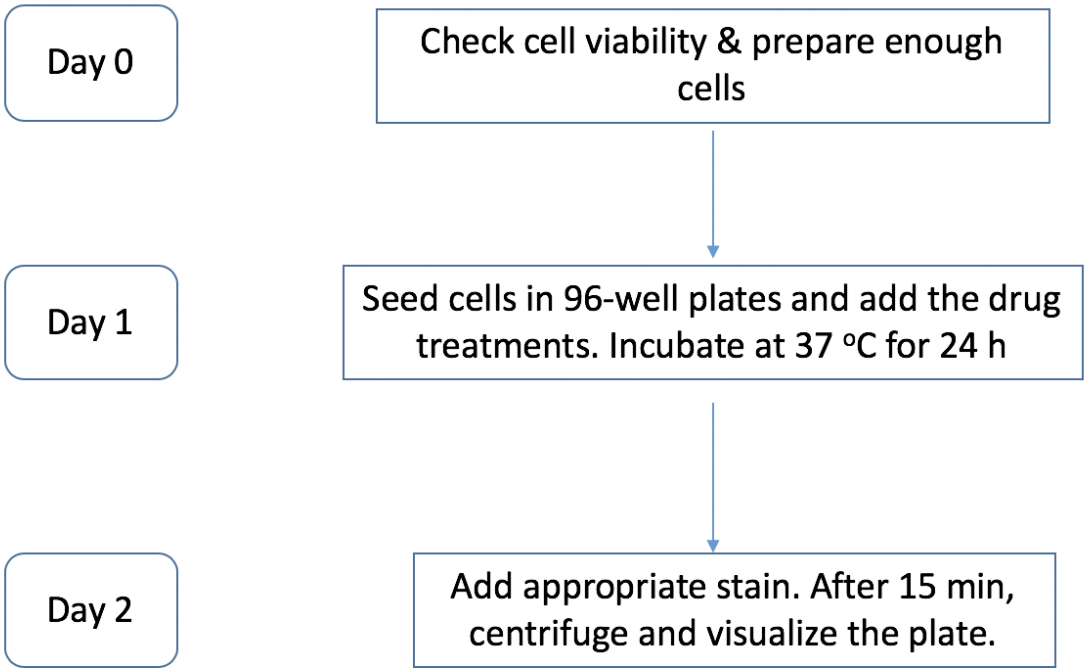
Fig. 1

Figure 1

A



B

Feature	Dye exclusion assays (TB, PI, EtBr, SYTOX etc.)	Assays monitoring a metabolic function (MTT, XTT, Alamar Blue, CKK-8 etc.)
Involvement of mitochondrial function	no	yes
Relatively low cost for high-throughput tests	yes, except SYTOX	no, except MTT
Assay requires higher drug dosages applied; estimated toxic dose	yes; LD50 (lethal dose)	no; IC50 (inhibitory dose)

Fig. 2

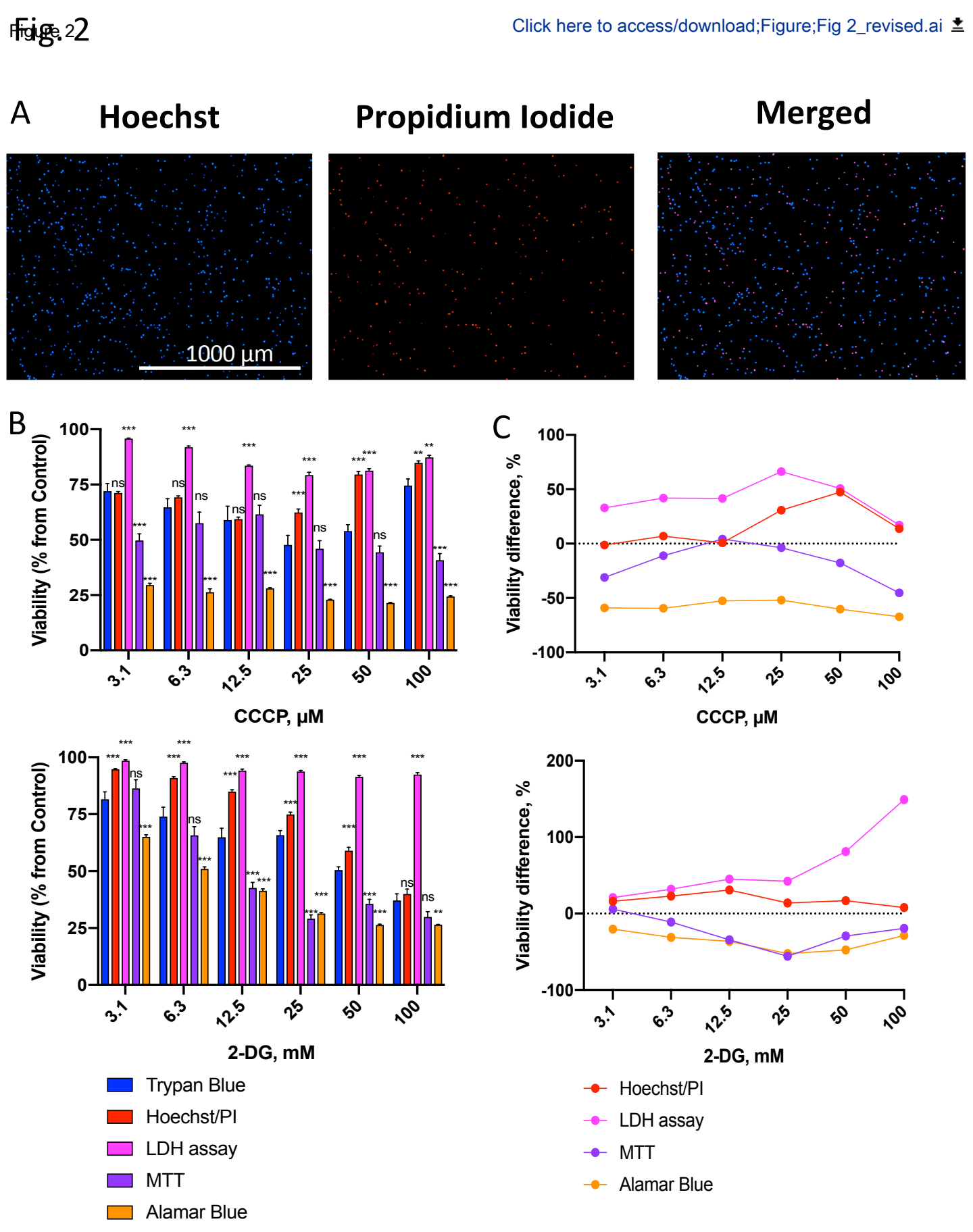


Fig. 3

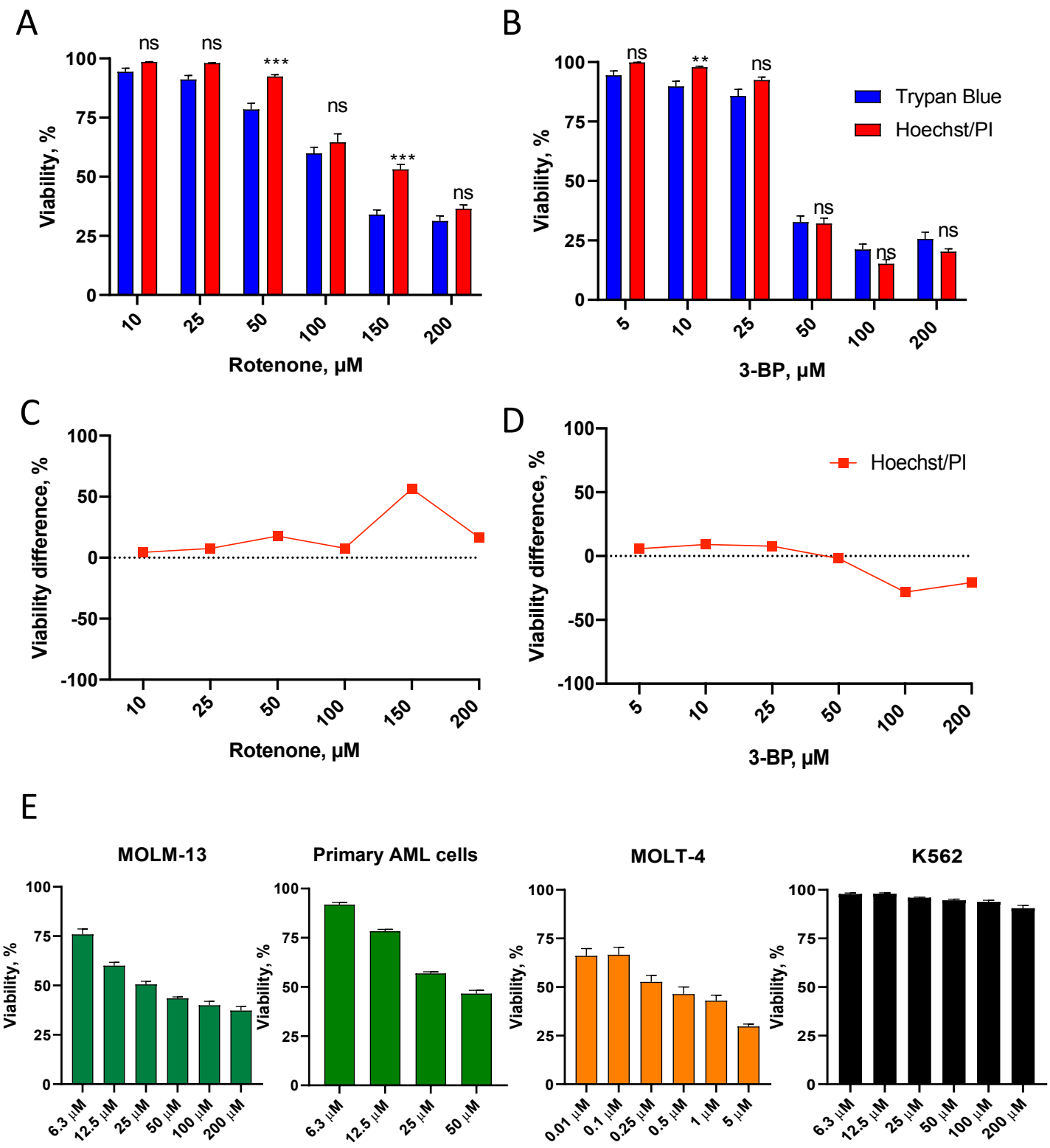


Fig. 4

A

3-BP, μM	Total cells, counts					
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6
0	3953	4438	4301	5013	4832	5049
6.3	3365	3506	4189	3078	3236	2463
12.5	2172	2918	3046	3020	3468	2550
25	1417	1493	1427	1188	1159	1049
50	887	799	848	834	822	913
100	636	554	618	586	558	564
200	568	638	564	572	618	633

B

3-BP, μM	Dead cells, counts					
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6
0	32	31	23	19	21	33
6.3	28	28	35	30	25	20
12.5	30	35	41	48	36	49
25	164	164	137	145	142	118
50	618	609	598	662	688	737
100	556	499	550	495	504	477
200	444	522	440	446	482	485

C

3-BP, μM	4 wells	6 wells
	% Viability	% Viability
6.3	99.80±0.08	99.82±0.07
12.5	99.27±0.16	99.24±0.31
25	89.50±1.12	89.28±0.99
50	26.22±4.67	23.45±5.70
100	12.34±2.45	12.44±2.64
200	21.15±1.90	21.71±1.77

D

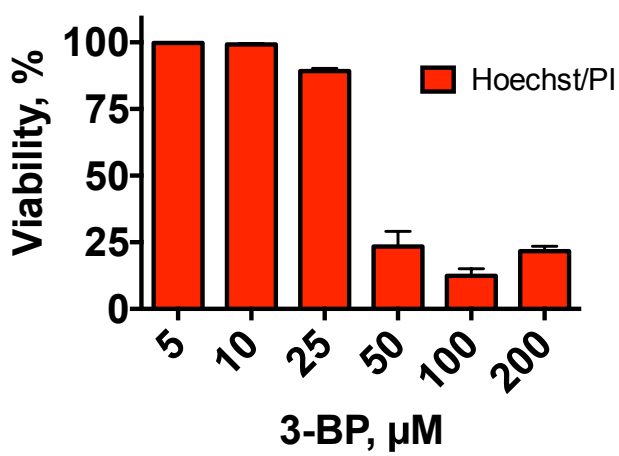


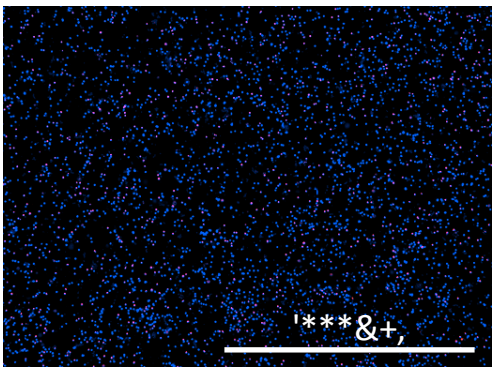
Figure 5

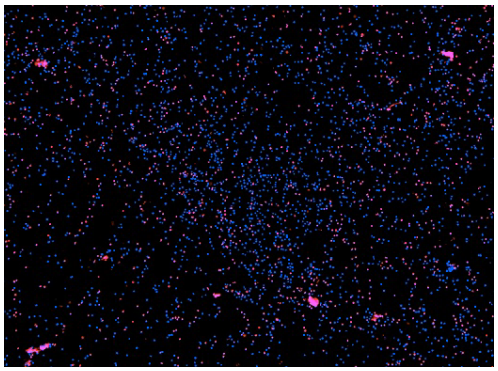
Fig. 5

Optimal **Sub-optimal**

A

PI concentration

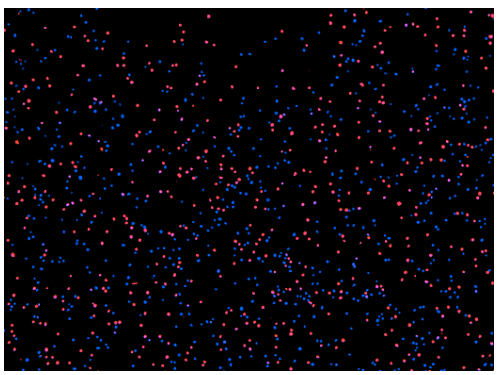


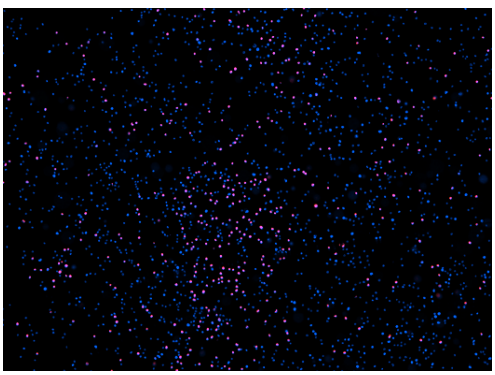


	Viability (%)
Normally stained	78.7
Overstained	61.6

B

Centrifugation

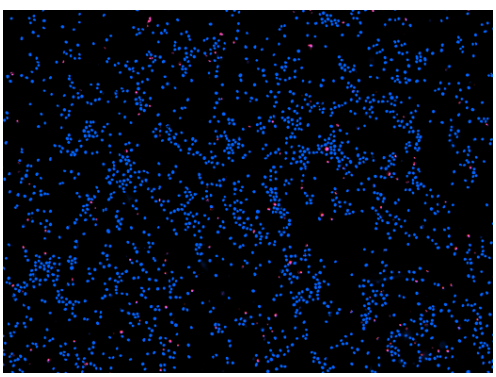


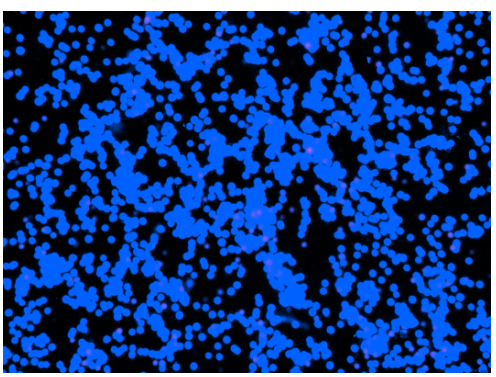


	Viability (%)
With centrifugation	54.6
Without centrifugation	80.1

C

Exposure time





	N, total cells
Normally exposed	1935
Overexposed	205

Table 1

Assay/dye	**Type(s) of cell death detected**	**Necessary equipment**
MTT, CKK-8, Alamar Blue (resazurin)	Apoptosis/Necrosis	Spectrophotometer
LDH release	Necrosis	Spectrophotometer
Trypan Blue (TB)	Apoptosis/Necrosis	Microscope
Acridine orange (AO)	Apoptosis/Necrosis/ Necroptosis	Fluorescence microscope
Hoechst 33342, DAPI	Apoptosis	Fluorescence microscope or flow cytometer
Propidium Iodide (PI)	Late apoptosis/Necrosis	Fluorescence microscope or flow cytometer

Key features
Inexpensive, rapid; endpoint assay; dependent on enzymes' activity (exclusively mitochondrial in case of MTT) and does not discriminate between modes of cell death[1,10]
Rapid, independent of mitochondrial enzymes' activity; expensive for high-throughput tests; detects necrotic cells with compromized plasma membrane[11,12]
Cell-impermeant; does not discriminate between modes of cell death; laborous and not suitable for high-throughput screening; more difficult to use with adherent cells; prone to subjective judgment of the user, but is considered the standard cell viability measurement method[13]
A nucleic acid dye with unique spectral properties, can distinguish between apoptosis and necrosis/necroptosis[14]
Cell-permeable; inappropriate on its own to monitor cell death; useful for co-staining; can be used to assess chromatin condensation and nuclei fragmentation in early apoptosis; can be paired with propidium iodide to distinguish apoptosis from necrosis[15,16]
Cell-impermeant intercalator; detects both late apoptosis and necrosis modes of cell death[17]. Toxic and permeable after long incubation times[18]

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Countess II automated cell counter	Thermo Fisher	
Cytation 5 Cell Imaging Multi-Mode Reader	BioTek	
RPMI-1640 Medium With L-glutamine and sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture	Sigma Aldrich	R8758-500ML
HyClone fetal bovine serum	GE Healthcare	#25-514
Penicillin-Streptomycin-Glutamine (100X)	Gibco	10378016
96-Well plates	Greiner Bio-One	655090
Hoechst 33342	Thermo Fisher	62249
Propidium Iodide	Thermo Fisher	50-596-072
2-Deoxy-D-glucose/2-DG	Chem-Impex	50-519-067
m-chlorophenylhydrazone/CCCP	Sigma Aldrich	C2759
Rotenone	Ark Pharm	AK115691
3-bromo-pyruvate	Alfa Aesar	1113-59-3
Pierce LDH assay kit	Thermo Fisher	50-103-5952
Alamar blue HS cell viability reagent (100mL)	Thermo Fisher	A50101
Thiazolyl blue tetrazolium bromide	ACROS Organics	AC158990010
Trypan blue stain (0.4%)	Gibco	15250-061
PBS tablets	Thermo Fisher	BP2944100
Cell lines		
OCI-AML2	ATCC	
MOLM-13	ATCC	
MOLT-4	ATCC	CRL-1582
K562	ATCC	CCL-243

	Comments/Description
Black or clear flat-bottomed 96-well plates	
20 mM solution; final concentration 1:1,000	
Dry powder; stock 1 mg/mL in PBS; final concentration 5 µg/mL (leukemia cells), 1 µg/mL (normal PBMCs)	
1 tablet + 200 mL of sterile water = 1x PBS solution	
AML cell line	
AML cell line	
ALL cell line	
CML cell line	

Editorial Requirements

- *Figures: 300 dpi, .ai/.psd*
  Saved as .ai, 300 dpi.
- *Tables: .xlsx*
  Done
- *Spelling*
  Checked
- *No commercial language (trademark symbols (™), registered symbols (®), and **company names** before an instrument or reagent).*
  Fulfilled
- *No personal pronounces (e.g., "we", "you", "our" etc.).*
  Fulfilled
- *What cell lines were used?*
  The list of cell lines used in the study is available in “Materials” table.

Reviewers' comments:
Reviewer #1:
Minor Concerns:

1. 3-Bromopyruvate and 2-deoxy-D-glucose are inhibitors of glycolysis. Although they decreases the viability of AML cells by impairing the energy supply, they could not be regarded as mitocans.

Thanks for highlighting this. As a matter of fact, we specifically used 2-DG as a non-mitocan (it is a glycolytic inhibitor) to show the robustness of this approach to measure cytotoxicity upon treatment with drugs having different mechanisms of action.

CCCP, rotenone, and 3-BP were used as examples of mitocans. There are many pieces of evidence that 3-BP impacts mitochondria, including 3-BP-mediated alkylation of mitochondrial dehydrogenases, increased mitochondrial ROS production, etc. [PMID 24842108]. In addition, 3-BP has been shown to inhibit mitochondrial OxPhos and other metabolic pathways, including LDH, PDH, TCA cycle, and glutaminolysis [PMID 30845728].

Reviewer #2:
Manuscript Summary:
This protocol demonstrates how to evaluate viability using PI and Hoechst staining with microscopy and imaging as with a cytation5. The argument being made is that traditional markers of viability assays that are often used in microscopy assays are dependant on mitochondrial function. The study was conducted only in an AML background with 1 cell line- OCR-AML2

Major Concerns:
1. Validity is only demonstrated in a single model system. I would like see the protocol

applied to other AML lines and primary samples. Given this is a challenge in cancer as a whole the applicability of the assay to other cancer or even other leukemia types would be important and should be demonstrated

We appreciate this suggestion. As a matter of fact, we have recently validated this protocol in wider panel of leukemia cell lines (AML: OCI-AML2, MOLM-13; ALL: MOLT-4, CCRF-CEM; CML: K562, KU812) and primary AML cells derived from AML patients (n = 12) [Panina SB, Pei J, Baran N, Konopleva M, Kirienko NV. Utilizing synergistic potential of mitochondria-targeting drugs for leukemia therapy. Front Oncol. 10:435. https://doi.org/10.3389/fonc.2020.00435].

That being said, the reviewer's concern was something that we could easily address, so we have included data here as well. Viability was determined for MOLM-13, K562, MOLT-4 cells, and a representative patient AML sample after treatment with rotenone (mitochondrial Complex I inhibitor) for 24 h. These data are in the updated Figure 3E and new Suppl. Fig. S1.

2. AML cells differentiate and may not die especially with mitochondrial dysfunction. We do not know here if the viability assays correlate with the differentiation of cells or mitocans directly. This should be demonstrated.

We appreciate the reviewer's concern. Unfortunately, we will be unable to address this point. The SARS-CoV-2 pandemic has resulted in a nearly complete shutdown of research at Rice University and MD Anderson. This precludes our ability to perform extra experiments to assess differentiation of AML cells. However, we made an attempt to assess relative sensitivity of leukemia cell lines to differentiation-inducing drugs using a bioinformatic approach based on data-mining of publicly available NCI-60 dataset.

First, from compounds analyzed by NCI-60, we chose drugs with published ability to induce differentiation: hexamethylene bisacetamide [PMID 19029824] and sodium butyrate [17331472]. Then, for these two drugs, we downloaded Drug activity Z-scores for every cell line from NCI-60 panel using the CellMiner™ tool: https://discover.nci.nih.gov/cellminer/.

Next, we calculated the sum of Z-scores of these two drugs per cell line. Since there are 6 leukemia cell lines in the NCI-60 panel (CCRF-CEM, HL-60, K562, MOLT-4, RPMI-8226, and SR), we calculated the average of this parameter (sum of Z-scores) between all 6 leukemia cell lines. In order to compare leukemia with other cancer types, a similar average parameter was defined for all other cancer cell lines (n = 54):

Cell line origin	Sum of Z-scores to Hexamethylene Bisacetamide and sodium butyrate, average between cell lines
LE: (n = 6)	5.13
All other (n = 54)	4.45

While this is by no means definitive, and a final answer would require validation by performing the experiment, these data do suggest that leukemia cells and other cancer cells have comparatively similar sensitivity to differentiation-inducing drugs.

3. External controls with no image-based validation should be presented- for viability Flow based assays (Annexin V and PI) in parallel and an assessment of OCR, in parallel with a differentiation assay and the other MTT, Trypan blue based exclusion assays in the presence and absence of Mitocan. This will illustrate the value and the effectiveness of these assays.

We appreciate this suggestion. Unfortunately, as noted above, we are currently unable to perform extra flow cytometry experiments. However, we validated this protocol in a wider panel of leukemia cell lines (AML: OCI-AML2, MOLM-13; ALL: MOLT-4, CCRF-CEM; CML: K562, KU812) and primary AML cells derived from AML patients (n = 12) [Panina SB, Pei J, Baran N, Konopleva M, Kirienko NV. Utilizing synergistic potential of mitochondria-targeting drugs for leukemia therapy. Front Oncol. 10:435. doi: 10.3389/fonc.2020.00435]. We included additional experimental information on these cell lines and primary cells. Please see updated Fig 3E and Suppl Figure S1.

4. Minor Concerns:
The figures are too small the quality is poor of the images.

The figures were mostly for reference. We have uploaded newer, high resolution images as .ai files. The quality should be much improved.

Reviewer #3:
Manuscript Summary:
Authors present a cytotoxicity assay based on HOECHST/PI staining which should be more suitable to detect the effect of mitocans since other frequently used methods (MTT, MTS,etc.) are based on mitochondrial activity of tested cells. Mitocans are potential therapeutical agents, their cytotoxic effect is recently often tested in wide range of tumors. They affect the fitness of mitochondrias and by that lead the cells to cell cycle arrest and/ or to apoptosis or other type of cell death. Authors tested this new type of cytotoxic method on suspension cells (AML cell line) in which assessment of fluorescence is more challenging. They showed reproducing results representing the ratio of viable/dead cells.

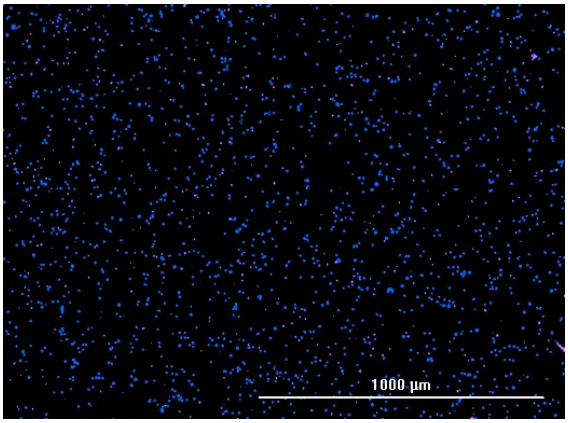
Major Concerns:
1. Number of cells in the well is one of the crucial steps to be optimized in this method. Authors in the discussion mentioned the optimal range to be 500-4000 cells per well.

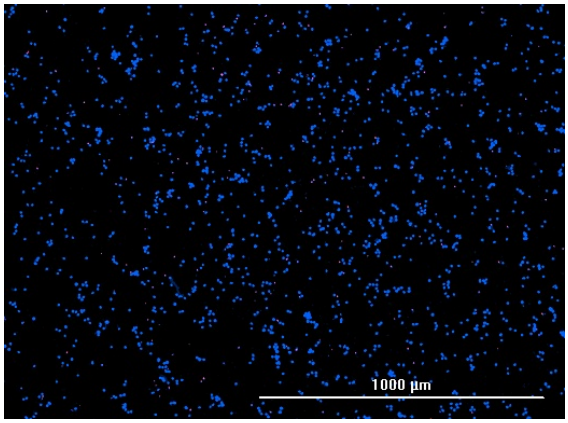
Thanks for highlighting this. We mentioned that 500-4000 cells were being captured in the center of a well depending on seeding density. The settings we used led to capturing images in the precise center of a well. Seeding density itself depends on 1) cell size: usually lower for large cells; 2) treatment duration: usually lower for longer

time-points; 3) division rate: usually lower for cell lines with fast division rate. We included these recommendations and specific examples in the protocol (please see Section 1.5 of the protocol).

2. In the same line, they mentioned some information about primary cells (in 2.1.2.) but it would be very beneficial to test how many cells from primary sample needs to be added into the well to achieve reliable and reproducing results.

We understand the reviewer's concern. Unfortunately, that is going to tend to be an empirically determined value. The fact is that primary cells tend to differ, since they are coming from patients. As such, we can realistically only offer guidelines, rather than a set rule. Typically, we seed 15,000-20,000 cells/well for primary AML samples and 40,000-50,000/well for primary normal PBMCs. This leads to sufficient cell counts for reproducible results (section 1.5). But, the exact density may need to be adjusted based on cell yield from the particular sample and/or number of experimental conditions. Please see below some examples of representative images showing untreated primary AML cells from different patients. Depending on the sample and the clinical treatment that has been received, viability in media/DMSO-control wells may range widely.



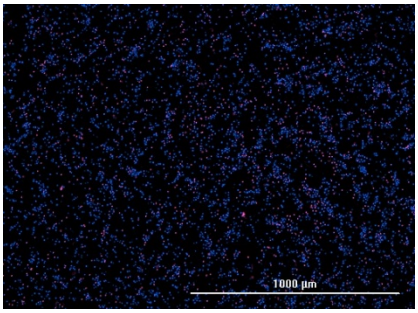


3. Section 2.1 What do you mean by empirically determined? Maybe it will be clearer with the video.

We appreciate this question. In general, empirically determined means that the experimenters may need to try a range of conditions to identify the drug concentration/ seeding density/etc in their own hands. Although the experiment is relatively robust, there will be any number of differences between labs(plastic ware, cell line, media composition, etc.) All of these factors will have subtle effects on the experiment, and may push the required dye concentration or cell seeding number higher or lower to achieve statistically accurate results. We find that a final concentration of 20 µM for the cell-permeant dye Hoechst 33342 works well with all of the cell types tested (leukemia

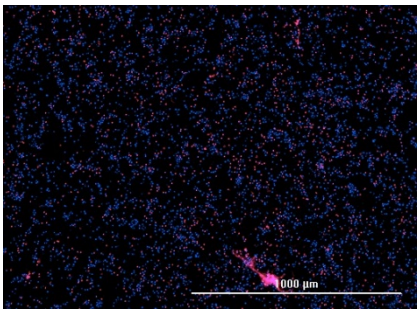
cells/normal blood cells). However, to determine optimal concentration for the cell-impermeant dye propidium iodide (PI), we tested different PI doses prior to experiments (i.e., this concentration was "empirically determined"): for instance, primary normal PBMCs were stained with PI of 1, 2.5, 5 µg/ml, and following viability was compared with Trypan Blue exclusion assay:

PBMC: untreated cells (DMSO-control):



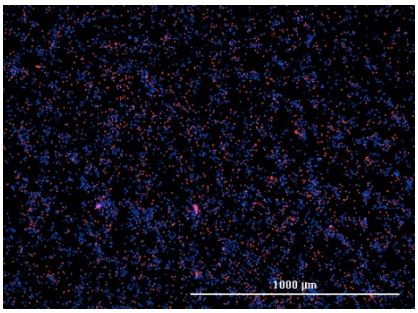
PI 1 µg/ml

Mean viability = 70%, closest to Trypan Blue estimation



PI 2.5 µg/ml

Mean viability = 51%



PI 5 µg/ml

Mean viability = 48%

We rephrased section 2.1 to make this more clear.

4. Another step which is crucial for precise and reproducing measurement is centrifugation. What parameters for centrifugation would they recommend for primary cells? I suppose it is different from cell line since these cells are usually smaller.

We used the same centrifugation parameters for AML cell lines, primary AML samples, and normal PBMCs (1000 rpm – 200 g, for 4 min). These centrifugation parameters are universally used with any cell types, both in conical tubes and in plates.

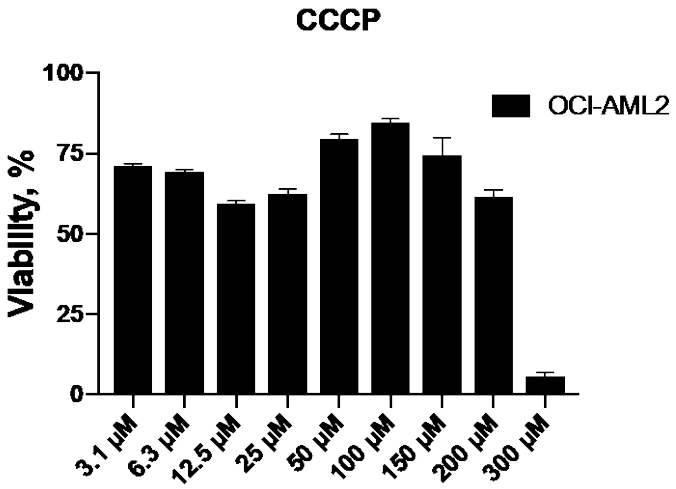
5. Note 2 in Section 2.4. Authors mentioned that the plate should be imaged after centrifugation as quickly as possible. Can they be more accurate? Are we talking about 1 minute, 5 or 15 minutes?

We included more specifics in Note 2 in Section 2.4. From our experience, we usually stain and centrifuge two full plates at a time (it's easier with regard to balancing and time management). After centrifugation, we immediately start imaging. Since it takes about 12 minutes/plate using our imaging protocol, the second plate is imaged within ~12-15 min after centrifugation. Anecdotally, we don't see a substantial difference within

30 min of centrifugation, but for best reproducibility, we would recommend keeping the centrifugation and imaging under 1 h.

6. Section Results: How the authors explain the effect of CCCP in higher concentrations? Why there were more viable cells (using HOECHST/PI or trypan blue) comparing to lower concentrations?

We added some discussion of this phenomenon to Representative Results section. We think it might be associated with precipitation of CCCP (which is hydrophobic) from the solution at higher doses, which would reduce the effective concentration and its impact on cells. We do note that increasing the CCCP dosage further reduced the viability farther. Hoechst/PI-estimated mean viability of OCI-AML2 cells also dropped down: at 150 µM - 74%, at 200 µM - 62%, at 300 µM – 6%. We're not sure what the cause of this phenomenon is. Perhaps the CCCP is having more than one effect on the cells.



7. In Fig.2 B authors compare MTT assay with trypan blue, HOECHST/PI and other methods. In MTT assay results are normalized to control untreated sample (absolute number of the cells) while HOECHST/PI and trypan blue show dead (viable) cells from actual number of the cells in the sample. Moreover, MTT assay is not apoptotic assay and should be interpreted differently. Therefore graphs in Fig. 2 are difficult to follow. How did the authors calculate the results of MTT assay to be able to plot them together with results from HOECHST/PI and trypan blue? The authors should comment on this and maybe rethink the visualization of the compared results from different methods. In my opinion in order to understand the effect of the drug it is necessary to include the information about the total number of the cells, not only percentage of dead or viable cells.

We appreciate this concern. Only the total number of cells can be measured using TB exclusion or Hoechst/PI method. We changed the title of the Y-axis (to Viability (% from Control)) in Figure 2B to point out that this is actually normalized viability plotted for each assay used. Normalized and absolute viability of immortalized cells usually does

not differ by much for trypan blue and Hoechst/PI assays. But for primary cells, this normalization is essential, because the viability of the controls ranges dramatically, depending on sample quality, thawing procedure etc. Moreover, since we compare each assay with the same trypan blue method, we think it is better to plot them in the same graph.

8. Minor Concerns:
1.6. ….from step 1.5. using……instead of 1.4. using.....

Thank you. It has been fixed.

Reviewer #4:
Manuscript Summary:
The paper describes a modified protocol that is based on differential staining with two fluorescent dyes, one of which is cell-permeant (Hoechst 33342) and the other is not (propidium iodide). The difference in staining can be combined with automated microscopy and image analysis. Many existing cytotoxicity assays, such as those based on tetrazolium salts or resazurin, require mitochondrial enzyme activity for accurate readout. Hence, these assays show reduced cell viability compared to methods independent of mitochondrial function. The method reported in this paper apparently circumvents this problem and the authors claim that it is particularly useful to accurately determine cell viability also in the presence of damage inflicted by compounds that target mitochondria, such as mitocans.

Major Concerns:
A). The Introduction should be more focused and must be re-orgainzed. Here follow main issues to be addressed.

1. Acute myeloid leukemia description appears somehow unrelated to the main topic of the protocol. This paragraph parhaps may be eliminated- A short description of this cancer type should be moved to the Methods (Representative results) section in order to introduce properly OCI-AML2 cell line.

Thank you for suggesting that. We moved a brief description of AML to the Representative results section.

2. The issue regarding the detection of cell viability after treatment with mitocans is, in my opinion, the key point of this protocol. Metabolic reprogramming in cancer cells is an emerging hallmark of cancer and a promising target for pharmaceutical treatment. But the hypothesis that "inflicting damage on mitochondria may slow down cell division and possibly even kill the cells" is quite simplistic. A number of comprehensive reviews have been written on this huge topic. Among others, Frontiers in Oncology (2017) 7, Article 295 and Biochimica et Biophysica Acta (2017) 1858, 686-699 deserve some discussion since, for example, both shift to glycolytic and respiratory metabolism may be found in

different cancer types. Thus, the introduction should stress how either increase or decrease in mitcohondrial functionality can significantly impinge on cell viability detection.

We looked at both suggested reviews and included relevant information in our Introduction.

3. What is the meaning of the statement at line 274-275: "Second, there are no red cells in the merged image, confirming that we are not seeing non-cells being labeled with PI."?

Thanks for pointing this out. We amended this statement as follows: "Second, since only cells being labeled with both Hoechst and PI are counted as dead (purple cells in the merged image), the possibility of counting debris is very low".

4. Fig. 1B, Explain what are the functions and chemical compositions of TB, PI, EtBr, SYTOX, MTT, XTT Alamar Blue etc. What does "Dependency upon enzyme activity" mean? Maybe the point is that some mitochondrial function is involved? Please, explain clearly.

We added this information to Figure 1 and its legend, and some was also included in the Introduction.

5. Fig 2A, a higher enlargement of the microphotographs should be shown for a better interpretation of the results.

Our imaging pipeline captures center of each well of 96-well plate at 4x magnification – in order to have more cells for counting and subsequent estimation of viability, than would be seen for 20x magnification, for instance. However, we have also provided all figures in .ai format, which should increase their resolution and quality.

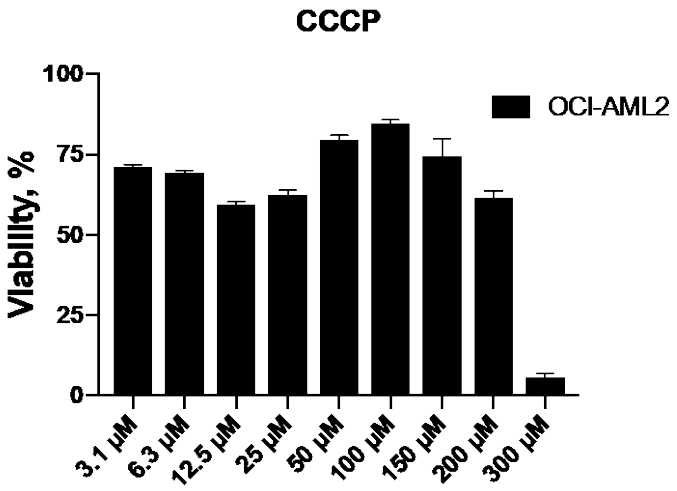
6. Whereas Trypan blue, MTT, Hoechst/PI and Alamar Blue assays eventually measure viable cells, LDH assay allows detection of necrotic/dead cells which is apparently very low or negligible. Thus, since y-axis corresponds to % viability, bars corresponding to LDH assay data should not be reported neither in the hystograms (Fig. 2B) nor in the plots (Fig. 2C). Perhaps, LDH assay data could be reported and discussed separately.

We appreciate the reviewer's comments. To clarify, LDH data in the previous version of the manuscript were plotted as cytotoxicity %, instead of viability %. Therefore, we have recalculated them as viability % = 100- cytotoxicity% for LDH assay. New graphs can be found in Figures 2B,C.

7. Comparison of data in Fig. 2B, 3A and 3B apparently shows that 2-DG, rotenone and

3-BP treatment causes OCI-AML2 cell death whereas treatment with the uncoupler CCCP is not toxic. Explain and discuss this result.

We added some discussion of this phenomenon to Representative Results section. We think it might be associated with precipitation of CCCP (which is hydrophobic) from the solution at higher doses, which would reduce the effective concentration and its impact on cells. We do note that increasing the CCCP dosage further reduced the viability farther. Hoechst/PI-estimated mean viability of OCI-AML2 cells also dropped down: at 150 µM - 74%, at 200 µM - 62%, at 300 µM – 6%. We're not sure what the cause of this phenomenon is. Perhaps the CCCP is having more than one effect on the cells.



8. Minor Concerns:
1. The title should be changed. As a suggestion: An automated differential nuclear staining assay for accurate determination of mitocan cytotoxicity.

Thank you for the suggestion. We changed the title of the manuscript.

2. References in table 1 should be included in the Reference list at the end of the manuscript and cited in the table with relevant citation numbers.

The reference list has been updated as suggested.

3. English language must be thoroughly checked.

Checked.

Fig. S1

Figure S1

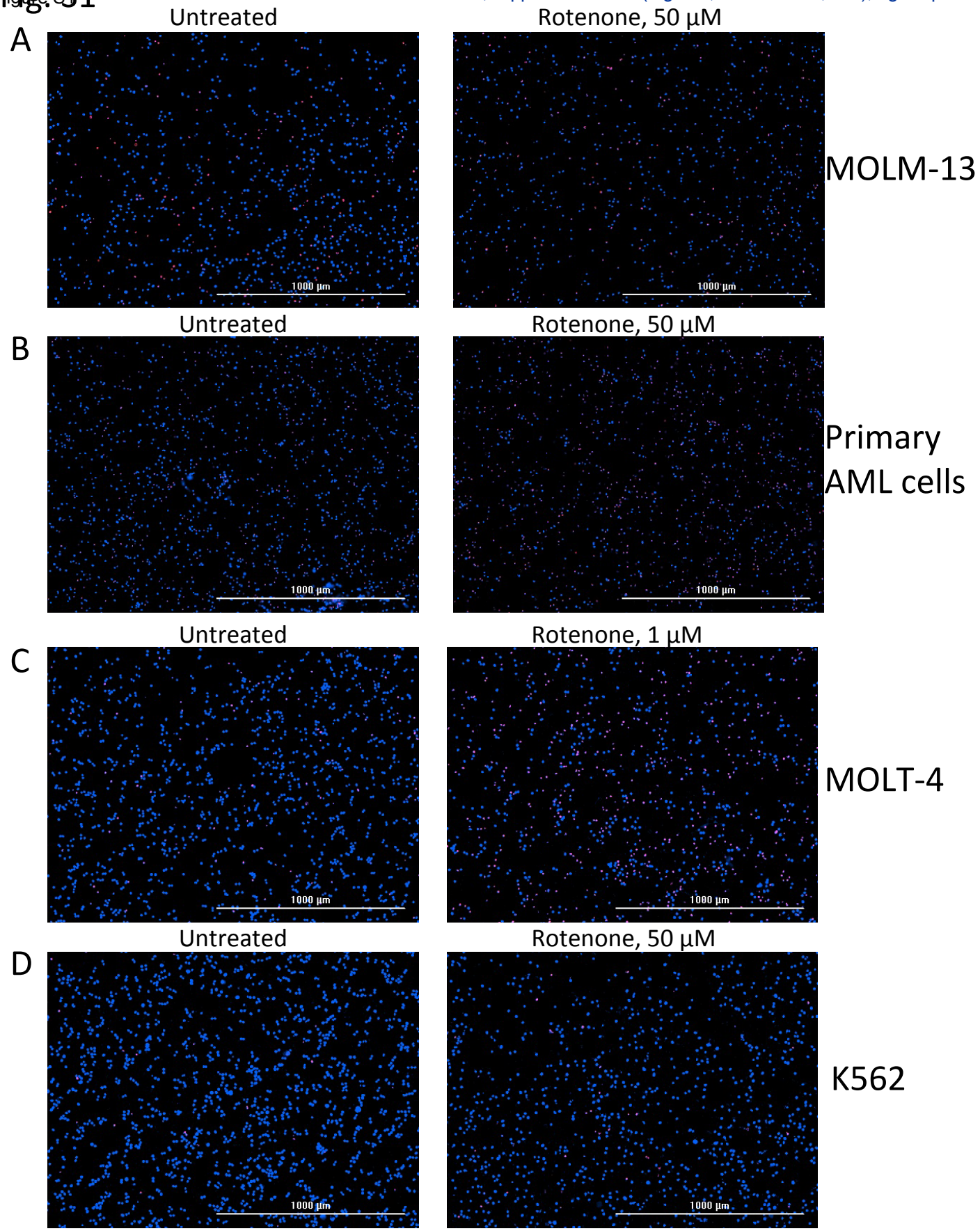


Table S1. Viability differences for a panel of assays in OCI-AML2 cells after CCCP treatment.

	Difference with trypan blue method, %		
CCCP, µM	**Hoechst/PI**	**LDH assay**	**MTT**
3.1	-1.19	33.00	-31.05
6.3	6.99	41.90	-11.03
12.5	0.71	41.60	4.31
25	30.73	66.33	-3.68
50	47.38	50.71	-17.71
100	13.81	17.12	-45.31
Median	**10.40**	**41.75**	**-14.37**

Alamar Blue
-59.04
-59.34
-52.52
-51.85
-60.27
-67.25
-59.19

Table S2. Viability differences for a panel of assays in OCI-AML2 cells after 2-DG treatment.

	Difference with trypan blue method, %		
2-DG, mM	**Hoechst/PI**	**LDH assay**	**MTT**
3.1	16.16	20.77	5.82
6.3	22.93	31.96	-11.13
12.5	30.85	45.07	-34.27
25	13.81	42.39	-55.79
50	16.96	81.12	-29.38
100	7.79	149.16	-19.37
Median	**16.56**	**43.73**	**-24.38**

Alamar Blue
-20.27
-31.11
-36.29
-52.29
-47.68
-28.67
-33.70

Table S3. Viability difference between Hoechst/PI and TB exclusion method in OCI-AML2 cells after roteno

Rotenone, µM	Viability difference, % (Hoechst/PI-trypan blue)
10	4.45
25	7.61
50	17.70
100	7.74
150	56.38
200	16.57
Median	**12.15**

ne treatment.

Supplementary Table S4. Viability difference between Hoechst/PI and TB exclusion method in OCI-A

3-BP, µM	Viability difference, % (Hoechst/PI-trypan blue)
5	5.73
10	9.00
25	7.72
50	-1.90
100	-28.23
200	-20.77
Median	**1.91**

ıML2 cells after 3-bromopyruvate (3-BP) treatment.

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Author(s):	Jingqi Pei, Svetlana B. Panina, Natalia V. Kirienko

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