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.

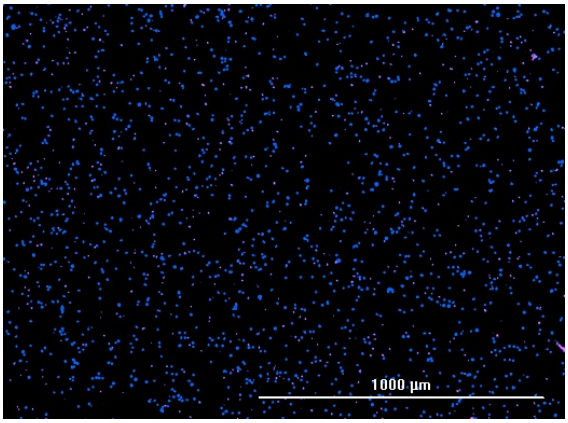
Major Concerns:
1. Number of cells in the well is one of the crucial steps to be optimized in this method. Authors in the discussion mentioned the optimal range to be 500-4000 cells per well.

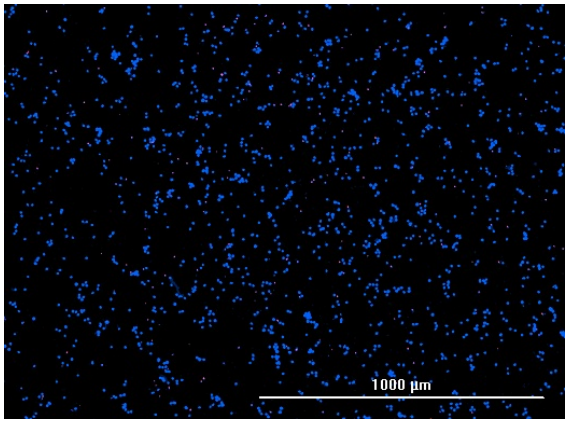
Thanks for highlighting this. We mentioned that 500-4000 cells were being captured in the center of a well depending on seeding density. The settings we used led to capturing images in the precise center of a well. Seeding density itself depends on 1) cell size: usually lower for large cells; 2) treatment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



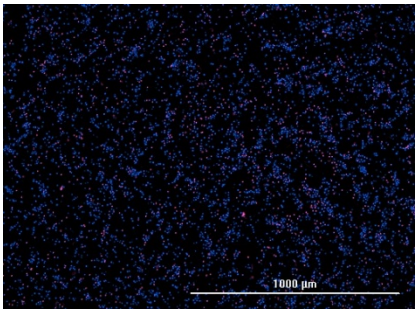


3. Section 2.1 What do you mean by empirically determined? Maybe it will be clearer with the video.

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

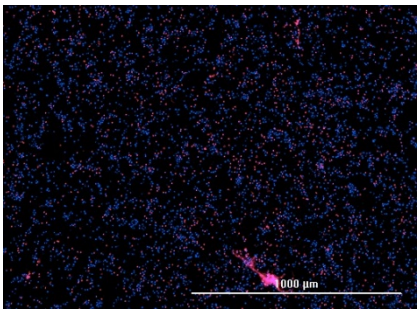
cells/normal blood cells). However, to determine optimal concentration for the cell-impermeant dye propidium iodide (PI), we tested different PI doses prior to experiments (i.e., this concentration was "empirically determined"): for instance, primary normal PBMCs were stained with PI of 1, 2.5, 5 µg/ml,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

PBMC: untreated cells (DMSO-control):



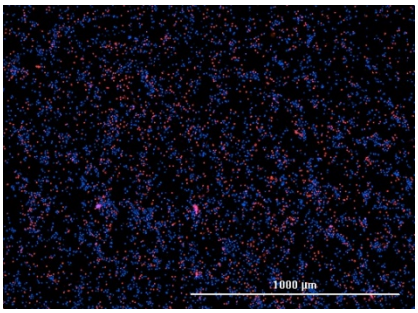
PI 1 µg/ml

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.

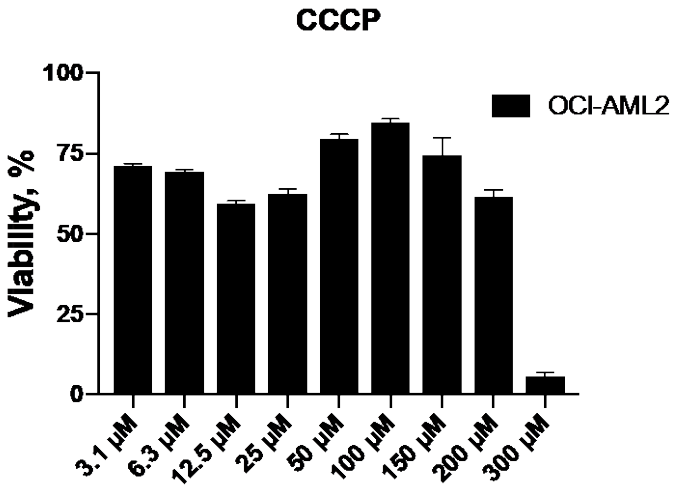
5. Note 2 in Section 2.4. Authors mentioned that the plate should be imaged after centrifugation as quickly as possible. Can they be more accurate? Are we talking about 1 minute, 5 or 15 minutes?

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.

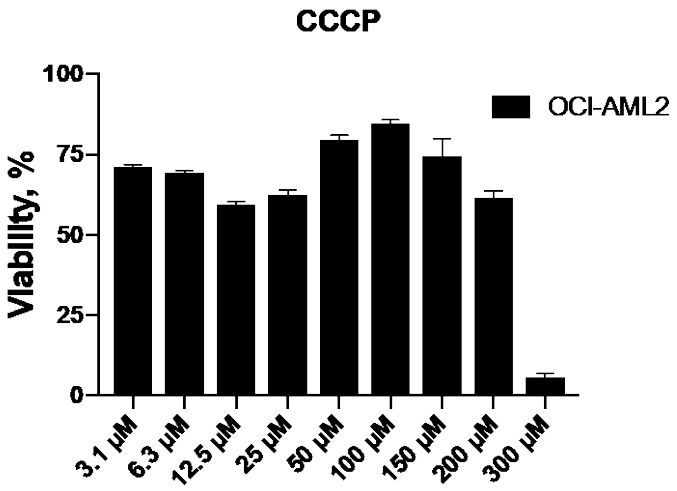
6. Whereas Trypan blue, MTT, Hoechst/PI and Alamar Blue assays eventually measure viable cells, LDH assay allows detection of necrotic/dead cells which is apparently very low or negligible. Thus, since y-axis corresponds to % viability, bars corresponding to LDH assay data should not be reported neither 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.