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

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

   If **Yes**, how far apart are the locations? **Filming will be in more than one room, but in the same building (same floor)**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Natalia Kirienko:** This method will allow researchers to assess cellular viability independently of mitochondrial function and it is amenable to high-throughput drug screening efforts.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Svetlana Panina:** This technique is fast, accurate, inexpensive, and allows the determination of compound cytotoxicity, including those impairing mitochondrial function, in most cell types.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Jingqi Pei:** This protocol is easy to perform. It is important to pay attention to drug preparation, treatment conditions, and cell density to ensure experimental accuracy and validity.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Svetlana Panina:** Visual demonstration of this method is important because the image acquisition and image analysis steps can be hard to recapitulate, especially for researchers having little experience with Gen5 software.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Cytotoxicity Assay Setup and Staining

2.1. Begin by preparing solutions of the compounds of interest at the desired concentration **[1]**. Always make sure to mix compounds by vortexing thoroughly **[a-added]**. To measure cytotoxicity of a single compound, prepare compounds at 2 X final concentration. If measuring cytotoxicity of compound combinations, prepare them at 4 X final concentration **[3]**.

2.1.1. WIDE: Establishing shot of talent diluting compounds.

2.1.1a Added shot: Talent mixes up compounds by vortexing.

2.1.2. Tube rack with labeled tubes containing the compounds of interest.

2.2. Prepare solvent-only controls by mixing the same amount of solvent with the appropriate medium **[1]**.

2.2.1. Talent preparing control solutions, with the media and solvent containers in the shot.

2.3. Collect cells into a 15-milliliter conical tube **[1]** and aliquot 10 microliters of the cell suspension for Trypan blue staining **[2]**. Add 10 microliters of 0.4% Trypan blue to the aliquot and use a hemocytometer or cell counter to count viable and non-viable cells **[3]**.

2.3.1. Talent collecting cells in the tube.

2.3.2. Talent transferring the aliquot to a microcentrifuge tube.

2.3.3. Talent adding Trypan blue to the tube and counting the cells with the Cell counter NOTE: shots 2.3.3 and 2.3.4 were combined into one

2.4. Pellet cells in the 15-milliliter tube at 200 x *g* for 5 minutes **[1]**, then aspirate or decant the supernatant **[2]**. Resuspend the cell pellet in assay-appropriate media at a cell density of 3 x 10^5 cells per milliliter **[3]**. *Videographer: This step is important!*

2.4.1. Talent putting the tube of cells in the centrifuge and closing the lid.

2.4.2. Talent aspirating the supernatant.

2.4.3. Talent resuspending the cells.

2.5. Use a multichannel pipette to seed 50 microliters of cell suspension into each well of a 96-well plate **[1]**. For single compound assays, add 50 microliters of 2 X compound solution into each well **[2]**. For solvent-control wells, add 50 microliters of test media containing the solvent at 2 X concentration **[3]**. *Videographer: This step is important!*

2.5.1. Talent adding cells to the 96-well plate.

2.5.2. Talent adding compound to the cells, with the compound solution in the shot and labeled.

2.5.3. Talent adding control to the cells, with the control solution in the shot and labeled.

2.6. For combination assays, add 25 microliters of each of the 4 X compounds into each well. For single compound-control wells, add 25 microliters each of 4 X compound solution and test medium. Make sure that the final concentration of DMSO does not exceed 0.5% **[1]**. *Videographer: This step is difficult and important!*

2.6.1. Talent adding the 4 X compound to the plate, with the compound solution in the shot and labeled.

2.7. **Jingqi Pei**: To ensure reproducibility, add drugs with the pipette tips touching the side of the wells, being careful to add the drugs to different locations. This step should be performed quickly, preferably taking no longer than 30 minutes per plate.

2.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

2.8. Gently tap the plate to mix the contents of the wells **[1]** and incubate it at 37 degrees Celsius and 5% carbon dioxide **[2]**. *Videographer: This step is important!*

2.8.1. Talent gently tapping the plate.

2.8.2. Talent putting the plate in the incubator and closing the door.

2.9. When ready to stain the cells with Hoechst 33342 and propidium iodide, prepare fresh 10 X staining solution **[1]** and add 10 microliters to each well **[2]**. Gently tap the plate **[3]** and incubate it at 37 degrees Celsius for 15 minutes **[4]**. *Videographer: This step is important!*

2.9.1. Talent preparing staining solution. **TEXT: CAUTION! Hoechst 33342 and propidium iodide dyes are potential carcinogens. Wear PPE.**

2.9.2. Talent adding staining solution to the cells.

2.9.3. Talent tapping the plate.

2.9.4. Talent putting the plate in the incubator and closing the door.

2.10. Centrifuge the plate at 200 x *g* for 4 minutes to bring all the cells to the bottom of the plate **[1]**. Then, carefully wipe the bottom of the plate with a damp laboratory wipe to remove any debris that may interfere with imaging **[2]**. Image the plate within 15 minutes after centrifugation **[3]**. *Videographer: This step is important!*

2.10.1. Talent opening the centrifuge and taking the plate out.

2.10.2. Talent wiping the bottom of the plate.

2.10.3. Talent putting the plate on the microscope stage.

3. Cytotoxicity Assay Data Acquisition

3.1. Open Gen5 software and create a new standard protocol. Click on **Procedure** and choose the type of plates to be used, typically 96-well black plastic plates **[1]**.

3.1.1. SCREEN: 61295_screenshot_1. 0:01 – 0:20.

3.2. Next, set the **Read** method as **Image**. Click **OK** and choose **DAPI** and **Texas Red** filter sets with a **4x** magnification objective. No offset is needed, since the wells' centers will be imaged **[1]**.

3.2.1. SCREEN: 61295_screenshot_2. 0:01 – 0:16.

3.3. For the DAPI filter, set LED to 10, integration time to 99, and gain to 0. For Texas Red, set LED to 8, integration time to 950, and gain to 18. Click on **Options** to make sure that the autofocus is performed on the DAPI channel and there is no offset in focusing between channels **[1]**.

3.3.1. SCREEN: 61295_screenshot_2. 0:20 – 0:52.

3.4. Save the protocol by clicking **OK**. Now images can be recorded using the **Read New** button **[1]**.

3.4.1. SCREEN: 61295_screenshot_2. 0:52 – 1:01.

3.5. Once images are recorded, click on **Data Reduction** and choose **Image Preprocessing**. Apply image preprocessing with dark background subtraction using **Auto** flattening size based on the DAPI signal. For Texas Red, use the same options as for channel 1. When finished, click **OK [1]**.

3.5.1. SCREEN: 61295_screenshot_3. 0:01 – 0:25.

3.6. Then, go to the **Cellular Analysis** panel and apply a nuclear mask based on the *Transformed DAPI signal* with a threshold value of 6000 units, minimal object size of 5 micrometers, and maximal object size of 25 micrometers. Analyze the entire image, exclude primary edge objects on the border of the image, and split touching objects **[1]**.

3.6.1. SCREEN: 61295_screenshot_3. 0:25 – 0:53.

3.7. In **Advanced detection options**, set the Rolling Ball diameter as 30 micrometers, and evaluate the background based on 5% of lowest pixels. Only keep the Cell Count in **Calculated Metrics [1].**

3.7.1. SCREEN: 61295_screenshot_3. 0:53 – 1:12.

3.8. After that, perform a **Subpopulation Analysis** based on the *Mean Transformed Texas Red signal* with a threshold value of 5000 units to count dead cells. Finally, access the resulting cell counts **[1]**.

3.8.1. SCREEN: 61295_screenshot_3. 1:12 – 1:43.

Results

4. Results: Validation of Hoechst/PI Cytotoxicity Assay in Leukemia Cells

4.1. Representative images of cells stained with Hoechst and Propidium Iodide are shown here **[1]**. The total number of cells is reasonably high **[2]** and greater than the number of dead cells **[3]**.

4.1.1. LAB MEDIA: Figure 2 A.

4.1.2. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the Hoechst image.*

4.1.3. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the PI image.*

4.2. When a panel of cytotoxicity assays was compared with Trypan blue exclusion, it was found that MTT and alamarBlue assays inaccurately measured cellular viability **[1]**. These mitochondrial enzyme-based assays showed significantly lower viability compared to Trypan Blue exclusion **[2]**.

4.2.1. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize the AlamarBlue and MTT bars in both graphs.*

4.2.2. LAB MEDIA: Figure 2 B.

4.3. Dual staining with Hoechst 33342 and PI had the best combination of robustness, sensitivity, and consistency with Trypan Blue staining **[1]** and the smallest median deviation from the Trypan Blue exclusion method after CCCP or 2-DG treatment **[2]**.

4.3.1. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize the red bars in both graphs.*

4.3.2. LAB MEDIA: Figure 2 C. *Video Editor: Emphasize the red circles and line in both plots.*

4.4. The Hoechst-PI assay was also effective at determining cellular viability after treatment with the mitochondria-targeting molecules rotenone and 3-bromopyruvate **[1]**. It was further validated with a panel of leukemia cell lines. These cells varied in rotenone sensitivity **[2]**, ranging from very sensitive **[3]** to resistant cells **[4]**.

4.4.1. LAB MEDIA: Figure 3 A – D.

4.4.2. LAB MEDIA: Figure 3 E.

4.4.3. LAB MEDIA: Figure 3 E. *Video Editor: Emphasize the MOLT-4 graph.*

4.4.4. LAB MEDIA: Figure 3 E. *Video Editor: Emphasize the K562 graph.*

4.5. Improper performance of the assay may compromise its accuracy. Several compromised outcomes are shown here **[1]**. Over-staining with PI **[2]**, neglecting the centrifugation step **[3]**, or overexposure in the Hoechst channel will cause sub-optimal results **[4]**.

4.5.1. LAB MEDIA: Figure 5.

4.5.2. LAB MEDIA: Figure 5 A.

4.5.3. LAB MEDIA: Figure 5 B.

4.5.4. LAB MEDIA: Figure 5 C.

Conclusion

5. Conclusion Interview Statements

5.1. **Jingqi Pei:** When attempting this protocol, remember to optimize the dye concentration and staining time for each cell line. Also, centrifugation of the sample plate is necessary to ensure imaging quality.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.9.1 and 2.10.1.*

5.2. **Svetlana Panina:** After identification of the compounds with cytotoxic effect on cancer cells, researchers may proceed with mechanistic studies to identify relevant targets.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

5.3. **Natalia Kirienko:** Screening small molecule libraries with this technique in parallel to conventional MTT assays will allow molecules that target mitochondrial function to be rapidly identified.

5.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.