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Title: Standardized Workflow for High-Content Imaging and Data Processing

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, but we cannot install screen recording software on the computer where our analysis software is located.**

***Videographer:** Please film the computer screen for all shots labeled as **SCREEN**, as the authors cannot install the screen recording software.*

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

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 16

Number of Shots: 52

Interviews

***Videographer:** Obtain headshots for all authors available at the filming location.*

Authors: The statements are edited for length and clarity. Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Xinyue Liu:** We focus on standardizing high-content imaging and comparing analysis methods to reliably quantify cell viability and drug responses.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**Describe the research gap or limitation in existing methods and how this protocol addresses them.**~~

- 1.2. **Xinyue Liu:** Existing imaging workflows vary in sample preparation, acquisition, and analysis, reducing reproducibility; our protocol standardizes these steps with defined quality controls.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**Describe the research contexts or systems in which this protocol can be applied.**~~

- 1.3. **Xinyue Liu:** This protocol applies to high-content pharmacological studies of adherent mammalian cells, particularly PI/Hoechst-based cell viability assays.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~**Describe the most important consideration or challenge when performing this protocol.**~~

- 1.4. **Xinyue Liu:** The key challenge is maintaining uniform cell seeding and consistent imaging parameters to ensure accurate, reproducible cell recognition and quantification.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Xinyue Liu**: Future studies can extend the workflow to other two-dimensional cell assays and develop analysis methods tailored to specific experimental objectives.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Sample Preparation and Staining Procedure

Demonstrator: Xinyue Liu

- 2.1. Begin by retrieving a T25 (*tee- twenty-five*) tissue culture flask containing HT22 (*H-tee- twenty-two*) cells from the 37 degrees Celsius incubator [1-TXT]. Next, check whether the cells have reached 80 percent confluence [2]. Discard the old medium [3], add 1 milliliter, 0.25% trypsin to the flask and incubate at room temperature for 1 to 2 minutes [4].
 - 2.1.1. WIDE: Talent placing a T25 culture flask containing HT22 cells inside the biosafety cabinet. **TXT: Human Skin Fibroblasts were used for the demonstration**
 - 2.1.2. SCOPE: Shot of healthy 80 percent confluent HT22 cells. **Author's NOTE: This is a video captured by a smartphone from the eyepiece by the videographer**
 - 2.1.3. CU: Talent aspirating the old medium from the culture flask.
 - 2.1.4. Talent adding 1 milliliter, 0.25 percent trypsin to cover the cells and leaving the flask at room temperature for 1 to 2 minutes.
- 2.2. Now, add complete DMEM (*dee-em-ee-em*) at three times the trypsin volume to stop the dissociation reaction [1]. Transfer the cell suspension to a 15-milliliter conical tube [2]. Centrifuge at 175 g for 3 minutes at room temperature [3]. Discard the supernatant and resuspend the pellet in 1 milliliter of complete DMEM to obtain a single-cell suspension [4].
 - 2.2.1. CU: Talent adding complete DMEM to the culture vessel to quench the trypsinization reaction.
 - 2.2.2. Talent transferring the cell suspension to a 15-milliliter conical tube.
 - 2.2.3. Talent placing the conical tube in the centrifuge.
 - 2.2.4. Shot of talent discarding the supernatant and resuspending the cell pellet in complete DMEM.

Videographer: Obtain CU shot of cell pellet as well to use as an inset.

- 2.3. Count the cells using a cell counter hemocytometer [1]. Then, seed 5,000 cells in 100 microliters of complete medium, per well of a 96-well plate [2] and incubate at 37 degrees Celsius with 5 percent carbon dioxide for 24 hours [3].
- 2.3.1. Shot of talent counting the cells using a cell-counting chamber. **Author's NOTE:** This segment only demonstrates the process of adding 10 microliters of diluted solution to the cell counting chamber.
- 2.3.2. CU: Talent adding cells in 100 microliters of complete medium into each well of a 96-well plate.
- 2.3.3. Talent placing the 96-well plate in a 37 degrees Celsius incubator.
- 2.4. Before staining, confirm that the cells are at 70% confluency, forming a single, well-dispersed monolayer [1]. To prepare the working dye solution, mix appropriate volumes of Propidium iodide and Hoechst [2 – TXT]. Discard the old medium [3] and wash each well twice with 100 microliters of PBS [4].
- 2.4.1. SCOPE: Shot healthy cells forming a monolayer at 70 percent confluency. **Author's NOTE:** This image was captured using the TL channel of the high-content instrument. Since only the confluence is demonstrated, the image clarity and contrast have not been adjusted.
- Videographer's NOTE:** For better presentation effect, the author changed the SCOPE shot in section 2.4.1 to a SCREEN shot, and the corresponding video file is named C4688.MP4.
- 2.4.2. Shot of talent preparing the dye solution **TXT: Propidium iodide - 6.684 µg/mL; Hoechst 33342 - 10 µg/mL** **Author's NOTE:** this segment only demonstrates the operation of mixing PI and Hoechst 33342 in proportion.
- 2.4.3. CU: Talent discarding the old medium from the wells of the 96-well plate.
- 2.4.4. CU: Talent washing each well with 100 microliters of PBS. **Author's NOTE:** Multiple angles of the washing operation were captured in this segment.
- Videographer:** Please obtain multiple reusable takes of 2.4.4, as this will be used again later in the protocol.
- 2.5. Add 100 microliters of the dye working solution [1] and incubate the plate at room temperature in the dark for 30 minutes [2]. After incubation, wash each well twice with 100 microliters of PBS [3], and then add 100 microliters of DMEM [4].
- 2.5.1. CU: Talent adding 100 microliters of dye working solution to each well.
- 2.5.2. Talent covering the plate to protect it from light and leaving it on the bench for 30 minutes.
- 2.5.3. Please reuse 2.4.4

2.5.4. CU: Talent adding 100 microliters of DMEM to each stained and washed well.

3. High-Content Imaging Setup

*Videographer: Please film the computer screen for all shots labeled as **SCREEN**, as the authors cannot install the screen recording software.*

- 3.1. Switch on the high-content imaging instrument and start the computer [1]. Open the instrument's analysis software [2]. Load the stained 96-well plate into the instrument [3] and select a field of view containing the samples for imaging [4].
 - 3.1.1. WIDE: Talent switching on the high-content imaging instrument and computer.
 - 3.1.2. SCREEN: Instruments' analysis software being opened.
 - 3.1.3. CU: Talent loading the stained 96-well plate into the imaging instrument.
 - 3.1.4. SCREEN: A sample-containing field of view being selected for imaging.

- 3.2. In the **Objective and Camera** settings, select **20XPh1SPlanFluorELWDADM** (*twenty-ex-pee-h-one-es-plan-floor-ee-el-w-d-a-d-m*) as the magnification, set Camera binning to **2**, and select **Widefield** acquisition mode [1]. In **Plate mode**, select the corresponding 96-well plate and adjust its parameters using the product dimensions [2]. Under **Site Option**, select **nine fields per well** and enable **laser-based focusing** [3]. In **Autofocus**, measure the bottom thickness of at least three wells, and enter the calculated average as the **Bottom thickness** [4]. Then, select **enable laser-based focusing** [5]. ~~set the allowable thickness variations—within individual wells, between adjacent wells, and across the entire plate, to assist accurate laser focusing.~~
 - 3.2.1. SCREEN: **20XPh1SPlanFluorELWDADM** objective being selected, camera binning being set to **2**, and **Widefield** acquisition mode being chosen.
 - 3.2.2. SCREEN: 96-well plate type being selected and the plate dimensions being entered in Plate mode.
 - 3.2.3. SCREEN: Under **Site Option**, **nine fields per well** being selected and **laser-based focusing** being enabled. **Author's NOTE: The enabling of laser-based focusing is not demonstrated in this segment.**

Query to Authors: Should these words be deleted from the narration too?

Author's Response: Yes, please delete these words from the narration

3.2.4. SCREEN: The average plate-bottom thickness value being entered

3.2.5. SCREEN: Autofocus variation parameters being adjusted. **Author's NOTE:** The shot has been changed to check "Enable laser-based focusing."

Query to Authors: The narration is changed to "Then, select enable laser-based focusing". **The old sentence is deleted from the narration. Please check if this is correct?**

Is 'enable laser-based focusing' the full name of the icon or the item?

Author's Response: This expression is correct. "enable laser-based focusing" is confirmed as the full official name of this option in the software.

3.3. Under **Wavelengths**, set the number of wavelengths to three [1]. For the first illumination, select **DAPI** (*dappy*) and choose **Auto Correction for FL** (*f-el*) under **Shading Correction** [2]. After locating the image, begin with an exposure of 20 to 30 milliseconds, adjusting it as required to obtain moderate fluorescence without overexposure [3]. Set the bit depth to **16** [4].

3.3.1. SCREEN: Number of wavelengths being set to three.

3.3.2. SCREEN: **DAPI** being selected as the first illumination, and **Auto Correction for FL** being chosen.

3.3.3. SCREEN: Initial exposure time being set between 20 and 30 milliseconds and the fluorescence image being previewed.

3.3.4. SCREEN: Exposure being adjusted as required and bit depth being set to 16.

3.4. Select **Laser with z-offset for focusing** and select **Use Z-stack**, select **Calculate Offset**, to choose an appropriate focal distance [1]. Enable **Digital Confocal** and set **Increase Sharpness and Reduce Noise** to **0.200** [2].

3.4.1. SCREEN: **Laser with z-offset** being selected and **Calculate Offset** being used to determine the focal distance.

3.4.2. SCREEN: **Digital Confocal** being enabled and **Increase Sharpness and Reduce Noise** being set to 0.200.

- 3.5. For the second illumination, select **Cy3** (*sigh-three*) and apply the previously established exposure and focus settings [1]. For the third illumination, select **TL 25** (*tee-el-twenty-five*) and adjust the exposure and focus in the same manner [2].

3.5.1. SCREEN: **Cy3** being set as the second illumination and previously determined exposure and focus settings being applied.

3.5.2. SCREEN: **TL 25** being set as the third illumination and previously determined exposure and focus settings being applied. **Author's NOTE:** The operation in this shot omits steps 3.4.1 to 3.4.2 of adjusting the exposure and focus in during the shoot as it is similar to previous steps.

Query to Authors: This part is deleted from the narration. Please check if this is correct. Or should we keep the narration of adjusting exposure and focus in the same manner as it was in the shot list earlier?

Author's RESPONSE: No need to delete it, please keep this part of the narration.

- 3.6. In the **Run** tab, name the plate using the format plate name, cell type, experiment ID, date, objective magnification, and imaging type [1]. Confirm the channel parameters under **Description** [2] and select **Acquire Plate** to begin imaging [3]. After acquisition is complete, close the software and switch off the instrument and computer [4].

3.6.1. SCREEN: The plate name according to the specified naming convention being entered along with experiment ID, objective magnification, and imaging type.

3.6.2. SCREEN: The channel parameters under **Description** being confirmed.

3.6.3. SCREEN: **Acquire Plate** being selected to begin imaging.

3.6.4. SCREEN: Automated plate acquisition in progress.

3.6.5. WIDE: Talent closing the software and switching off the imaging instrument and computer.

4. Live–Dead Image Processing and Analysis

- 4.1. Open the instrument analysis software, select **Review Plate**, and then select **Plate** [1]. In **Date** view, choose **Well Arrangement** and select a field of view from the positive group as a reference [2]. Finally, under **Run Analysis**, select the **Live–Dead processing** program [3].

4.1.1. SCREEN: Analysis software being opened and **Review Plate** being selected followed by **Plate**. **Author's NOTE:** Due to the involvement of computer passwords, this segment has been divided into two separate videos: opening the analysis software and selecting "Review Plate" followed by "Plate."

Videographer's NOTE: this step was split into two video recordings, one of which is C4674.MP4 and the other is C4676.MP4.

- 4.1.2. SCREEN: **Well arrangement** in **Date** view being selected and a positive-group field of view being chosen as the reference
- 4.1.3. SCREEN: **Run Analysis** being opened and the **Live–Dead** processing program being selected.
- 4.2. Configure **Wavelength 1** and **Wavelength 2** sequentially by first setting the source to **DAPI** under **Wavelength 1** parameters and retaining the **Standard** algorithm [1]. Set **Stained Cell** type to **All Cells** and **Stained Area** to **Nucleus** [2]. Set **Approximate Minimum Width** to 8 to 10 micrometers, **Approximate Maximum Width** to 30 to 35 micrometers and **Intensity above local background** to 2,000 to 10,000 gray levels [3]. Enable **Split Touching Objects** and use **Preview** to assess object recognition [4].
 - 4.2.1. SCREEN: **Wavelength 1** Source being set to **DAPI** and **Standard** algorithm being selected.
 - 4.2.2. SCREEN: Stained cell type being set to **All Cells** and Stained area to **Nucleus**.
 - 4.2.3. SCREEN: **Minimum width**, **Maximum width**, and **intensity-above-local-background** parameters being set.
 - 4.2.4. SCREEN: **Split Touching Objects** being enabled and **Preview** being selected to inspect the recognition mask.
- 4.3. Under **Wavelength 2** Parameters, set the source to **Cy3** [1]. Set **Stained Cell** type to **Dead** and **Stained Area** to **Nucleus** [2]. Apply the same **Minimum Width**, **Maximum Width**, and **Intensity-above-local-background** ranges used for **Wavelength 1** [3]. Open **Configure Summary Log**, and select the parameters required for data output [4]. Perform a **Test Run**, assess the recognition results, and save the settings when the recognition is satisfactory [5].
 - 4.3.1. SCREEN: **Wavelength 2** Source being set to **Cy3**.
 - 4.3.2. SCREEN: Stained cell type being set to **Dead**, Stained area to **Nucleus**.
 - 4.3.3. SCREEN: Same object-width and intensity parameters being set, as before.
 - 4.3.4. SCREEN: **Configure Summary Log** being opened and selecting the required output parameters.
 - 4.3.5. SCREEN: A **Test Run** being performed, recognition results being reviewed, and settings being saved.

Results

5. Results

- 5.1. Using Propidium iodide plus Hoechst 33342 (*hoekst – three-three-three-four-two*) double staining, the Live Dead module identified all nuclei in the DAPI channel and dead-cell nuclei in the Cy3 channel [1]. Its marking result was reported to be generally comparable with those obtained using Multi-Wavelength Cell Scoring [2], CME [3], and ImageJ [4].
- 5.1.1. LAB MEDIA: Figure 3. *Video Editor: Please highlight image B and add arrows pointing to the green and red cells, labeling them “Live” and “Dead,” respectively*
- 5.1.2. LAB MEDIA: Figure 3. *Video Editor: Please highlight image A and add arrows pointing to the grey and red cells, labeling them “Live” and “Dead,” respectively*
- 5.1.3. LAB MEDIA: Figure 3. *Video Editor: Please highlight image C and add arrows pointing to the yellow and blue cells, labeling them “Live” and “Dead,” respectively*
- 5.1.4. LAB MEDIA: Figure 3. *Video Editor: Please simultaneously highlight images D and E, labeling them “All Cells” and “Dead Cells,” respectively.*
- 5.2. Across nine fields of view, no significant differences were reported among Live Dead, the other evaluated processing methods, and traditional manual counting for total cell count [1], dead-cell count [2], or the dead-to-all-cell ratio [3]. Across 81 fields of view, no significant difference was reported for the dead-cell-area ratio [4], although the average fluorescence-intensity ratio differed significantly among the various instrument-based methods [5].
- 5.2.1. LAB MEDIA: Figure 5. *Video Editor: Please highlight all the bars in Graph A and the ‘Total Cell Count’ y-axis label at the same time.*

- 5.2.2. LAB MEDIA: Figure 5. **Video Editor:** Please highlight all the bars in Graph **B** and the 'Dead Cell Count' y-axis label at the same time.
- 5.2.3. LAB MEDIA: Figure 5. **Video Editor:** Please highlight all the bars in Graphs **C** and Graph **D** and the 'Dead/Total Cell Count' y-axis label at the same time.
- 5.2.4. LAB MEDIA: Figure 5. **Video Editor:** Please highlight all the bars in Graph **E** and the 'Dead/Total Cell Area' y-axis label at the same time.
- 5.2.5. LAB MEDIA: Figure 5. **Video Editor:** Please highlight each bar in Graph **F** at the same time in a different color, especially emphasizing the higher ImageJ fluorescence-intensity ratio.

- 1. Fibroblasts
Pronunciation link: <https://www.merriam-webster.com/dictionary/fibroblast>
IPA: /'faɪbrəˌblæsts/, /'fɪbrəˌblæsts/
Phonetic Spelling: FY-bruh-blasts / FIB-ruh-blasts
- 2. Confluence
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluence>
IPA: /'kɑːnˌfluːəns/, /kənˈfluːəns/
Phonetic Spelling: KAHN-floo-uhns / kuhn-FLOO-uhns
- 3. Trypsin
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /'trɪpsɪn/
Phonetic Spelling: TRIP-sin
- 4. Dissociation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>
IPA: /,dɪˌsoʊsi'eɪʃən/, /,dɪˌsoʊʃi'eɪʃən/
Phonetic Spelling: dih-soh-see-AY-shuhn / dih-soh-shee-AY-shuhn
- 5. Conical
Pronunciation link: No confirmed link found
IPA: /'kɑːnɪkəl/
Phonetic Spelling: KAH-ni-kuhl
- 6. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sentrəˌfjuːdʒ/
Phonetic Spelling: SEN-truh-fyooj
- 7. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /,suːpərˈnɛtənt/
Phonetic Spelling: soo-per-NAY-tuhnt
- 8. Monolayer
Pronunciation link: <https://www.merriam-webster.com/dictionary/monolayer>

- IPA: /'mɑ:nouˌleɪər/
Phonetic Spelling: MAH-noh-lay-er
9. Propidium
Pronunciation link: No confirmed link found
IPA: /proʊˈpɪdiəm/
Phonetic Spelling: proh-PID-ee-uhm
10. Iodide
Pronunciation link: No confirmed link found
IPA: /'aɪəˌdaɪd/
Phonetic Spelling: EYE-uh-dyde
11. Hoechst
Pronunciation link: No confirmed link found
IPA: /hɜːkst/
Phonetic Spelling: HURKST
12. Trypsinization
Pronunciation link: No confirmed link found
IPA: /ˌtrɪpsɪnɪˈzeɪʃən/
Phonetic Spelling: trip-sin-ih-ZAY-shuhn
13. Magnification
Pronunciation link: No confirmed link found
IPA: /ˌmæɡnəfɪˈkeɪʃən/
Phonetic Spelling: mag-nuh-fih-KAY-shuhn
14. Widefield
Pronunciation link: No confirmed link found
IPA: /'waɪdˌfiːld/
Phonetic Spelling: WYDE-feeld
15. Autofocus
Pronunciation link: No confirmed link found
IPA: /ˌɔːtoʊˈfoʊkəs/
Phonetic Spelling: aw-toh-FOH-kuhs
16. Wavelengths
Pronunciation link: No confirmed link found
IPA: /'weɪvˌlɛŋkθs/
Phonetic Spelling: WAYV-lengths
17. DAPI
Pronunciation link: No confirmed link found
IPA: /'dæpi/
Phonetic Spelling: DAP-ee
18. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊˈresəns/, /flɔːˈresəns/
Phonetic Spelling: floo-RESS-uhns / flaw-RESS-uhns
19. Overexposure
Pronunciation link: No confirmed link found

- IPA: /ˌoʊvərɪkˈspoʊzər/
Phonetic Spelling: oh-ver-ik-SPOH-zher
20. Confocal
Pronunciation link: No confirmed link found
IPA: /kɑːnˈfoʊkəl/
Phonetic Spelling: kahn-FOH-kuhl
21. Acquisition
Pronunciation link: <https://www.merriam-webster.com/dictionary/acquisition>
IPA: /ˌækwəˈzɪʃən/
Phonetic Spelling: ak-wuh-ZISH-uhn
22. Nucleus
Pronunciation link: No confirmed link found
IPA: /ˈnuːkliəs/
Phonetic Spelling: NOO-klee-uhs
23. Algorithm
Pronunciation link: <https://www.merriam-webster.com/dictionary/algorithm>
IPA: /ˈælgəˌrɪðəm/
Phonetic Spelling: AL-guh-rith-uhm
24. Micrometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /ˈmaɪkroʊˌmiːtərz/
Phonetic Spelling: MY-kroh-mee-terz
25. Nuclei
Pronunciation link: No confirmed link found
IPA: /ˈnuːkliˌaɪ/
Phonetic Spelling: NOO-klee-eye
26. ImageJ
Pronunciation link: No confirmed link found
IPA: /ˈɪmɪdʒ dʒeɪ/
Phonetic Spelling: IM-ij-jay
27. Fluorescence-intensity
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊˈresəns ɪnˈtensəti/, /flɔːˈresəns ɪnˈtensəti/
Phonetic Spelling: floo-RESS-uhns in-TEN-suh-tee / flaw-RESS-uhns in-TEN-suh-tee