

FINAL SCRIPT: APPROVED FOR FILMING



Submission ID #: 71863

Scriptwriter Name: Ruchira Mukherji

Project Page Link: https://review.jove.com/files_upload.php?src=21918263

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.

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~~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**

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.

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- 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 Propidium iodide and Hoechst 33342 [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.

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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.
 - 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."
- 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

Commented [PG3]: Authors: Should these words be deleted from the narration too?

Commented [PG4]: 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?

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.
- 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.

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- 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 **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: /ˈfaɪbrəˌblæsts/, /ˈfɪbrəˌblæsts/
Phonetic Spelling: FY-bruh-blasts / FIB-ruh-blasts
 2. Confluence
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluence>
IPA: /ˈkɑːnˌfluːəns/, /kənˈfluːəns/
Phonetic Spelling: KAHN-floo-uhns / kuhn-FLOO-uhns
 3. Trypsin
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /ˈtrɪpsɪn/
Phonetic Spelling: TRIP-sin

4. Dissociation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>
IPA: /ˌdɪˌsoʊsiˈeɪʃən/, /ˌdɪˌsoʊʃiˈeɪʃən/
Phonetic Spelling: dih-soh-see-AY-shuhn / dih-soh-shee-AY-shuhn
5. Conical
Pronunciation link: No confirmed link found
IPA: /ˈkɑːnɪkəl/
Phonetic Spelling: KAH-ni-kuhl
6. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: SEN-truh-fyooj
7. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈneɪtənt/
Phonetic Spelling: soo-per-NAY-tuhnt
8. Monolayer
Pronunciation link: <https://www.merriam-webster.com/dictionary/monolayer>
IPA: /ˈmɑːnoʊˌleɪər/
Phonetic Spelling: MAH-noh-lay-er
9. Propidium
Pronunciation link: No confirmed link found
IPA: /ˈproʊˈpɪdiəm/
Phonetic Spelling: proh-PID-ee-uhm
10. Iodide
Pronunciation link: No confirmed link found
IPA: /ˈaɪəˌdaɪd/
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: /ˌmæɡnəfiˈkeɪʃən/
Phonetic Spelling: mag-nuh-fih-KAY-shuhn
14. Widefield
Pronunciation link: No confirmed link found
IPA: /ˈwaɪd fiːld/
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: /ˈweɪvˌlɛŋkθs/
Phonetic Spelling: WAYV-lengths
17. DAPI
Pronunciation link: No confirmed link found
IPA: /ˈdæpi/
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: /ˌoʊvərɪkˈspoʊʒər/
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: /'ɪmɪdʒ dʒeɪ/

Phonetic Spelling: IM-ij-jay

27. Fluorescence-intensity

Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>

IPA: /flʊ'reɪəns ɪn'tensəti/, /flɔ:'resəns ɪn'tensəti/

Phonetic Spelling: floo-RESS-uhns in-TEN-suh-tee / flaw-RESS-uhns in-TEN-suh-tee