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Title: A Cost-Effective Workflow for Mono- and Multiplex Immunofluorescence in Adherent Two-Dimensional Cell Cultures on Glass Coverslips

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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? **No.**
- 3. Filming location:** Will the filming need to take place in multiple locations? **Yes.**
If **Yes**, how far apart are the locations? **50 meters.**
- 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: 12

Number of Shots: 33

Interviews

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

INTRODUCTION:

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

- 1.1. **Jean Luz:** Our research focuses on developing gene therapies for cancer and using fluorescence imaging to visualize proteins involved in activating antitumor immune responses.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.2. **Jean Luz:** This protocol allows researchers to examine the subcellular localization of multiple targets within the same cell and generate images for further analysis.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

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

- 1.3. **Jean Luz:** A key consideration is careful panel design, as repeated host species and overlapping fluorophore spectra can compromise accurate signal interpretation.
 - 1.3.1. 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.~~

- 1.4. **Jean Luz:** Future studies can build on this work by using post-acquisition image analysis to determine target localization and infer protein function within cellular compartments.
 - 1.4.1. 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. Immunofluorescence Staining

Demonstrator: Jean Luz

2.1. Begin by arranging the materials required for cell culture inside a biosafety cabinet [1]. Using sterile forceps, transfer one sterile glass coverslip into each designated well of a 24-well tissue culture plate [2]. Seed 0.5 milliliters of cell suspension per well [3-TXT] and incubate cells at 37 degrees Celsius with 5% carbon dioxide until the desired confluence is reached [4].

2.1.1. WIDE: Talent arranging sterile cell culture materials inside a biosafety cabinet.

2.1.2. CU: Talent transferring the sterile glass coverslips into a 24-well tissue culture plate

2.1.3. Talent transferring 0.5 mL cell suspension per well **TXT: Seeding density: 3×10^4 – 5×10^4 cells/well**

2.1.4. Talent placing the plate at 37 °C

2.2. Once the cells have reached the desired confluence, aspirate the culture medium [1] and gently rinse the cells once with 0.5 milliliters of 1x (*one-x*) TBS, per well [2]. Add 0.5 milliliters of 4 percent paraformaldehyde fixative in PBS per well and incubate for 10 minutes at room temperature [3]. Aspirate the paraformaldehyde [4], then wash the cells three times per well with TBS [5].

2.2.1. Talent aspirating the culture medium

2.2.2. Talent rinsing the cells with 0.5 mL of TBS

2.2.3. Talent adding 0.5 mL of 4% paraformaldehyde and keeping the plate aside for 10 minutes

2.2.4. Talent aspirating the paraformaldehyde

2.2.5. Talent washing the cells with TBS

2.3. Skip permeabilization for membrane proteins, and for intracellular or nuclear protein staining, permeabilize the cells with 0.5 milliliters of 0.1 to 0.3% non-ionic detergent in TBS per well for 5 to 10 minutes at room temperature [1]. Wash the cells 3 times with TBS containing 0.05 percent Tween 20, or TBST (*T-B-S-T*) [2]. Add 0.5 milliliters of

blocking solution containing 5 percent BSA in TBS per well and incubate for 30 minutes at room temperature [3].

2.3.1. Talent adding 0.5 mL of 0.1–0.3% non-ionic detergent in TBS to each well

2.3.2. Talent washing the cells with TBST

2.3.3. Talent adding 0.5 mL of the blocking solution in each well and keeping the plate aside for 30 minutes

2.4. Aspirate the blocking buffer [1] and prepare the primary antibody panels in TBST containing 1% BSA [2]. For multiplex immunofluorescence, combine the unconjugated primary antibodies from different host species, targeting distinct antigens, into a single antibody cocktail [3].

2.4.1. Talent aspirating the blocking buffer

~~2.4.2. TEXT ON A PLAIN BACKGROUND:~~

~~Primary antibodies against distinct antigens:~~

~~Anti-p14ARF~~

~~Anti-actin~~

~~Anti-interferon- β~~ NOTES: Removed as it was not necessary

2.4.3. Talent preparing the primary antibody solution with TBST

2.4.4. Talent combining the primary antibodies into a single antibody cocktail.

2.5. Add 150 microliters of the primary antibody solution to each well, ensuring the coverslips are completely covered, while excluding the no-antibody and no-primary control wells. Incubate in a humidified chamber at room temperature for 1.5 hours [1]. Wash the cells three times with TBST for 3 minutes each at room temperature, aspirating the wash buffer after each wash [2].

2.5.1. Talent adding 150 μ L of the primary antibody cocktail to each well and keeping the plate aside for 1.5 hours

2.5.2. Talent washing the cells three times with TBST and aspirating the wash buffer after each wash.

2.6. Prepare the secondary antibody solution in TBST containing 1% bovine serum albumin [1]. For multiplex immunofluorescence, combine the secondary antibodies into a single antibody cocktail according to the host species of the primary antibodies [2]. Add 150 microliters of the antibody solution per well, ensuring the coverslips are completely covered while excluding the no-antibody control, and incubate in the dark for 1 hour at

room temperature [3]. Wash the cells three times with TBST for 3 minutes each at room temperature, aspirating the wash buffer after each wash [4].

- 2.6.1. Talent preparing secondary antibody dilutions with TBST
- 2.6.2. Talent combining the secondary antibodies into a single antibody cocktail
- 2.6.3. Talent adding 150 µL of the secondary antibody cocktail and keeping the plate aside in the dark after wrapping in aluminium foil
- 2.6.4. Talent washing the cells three times with TBST and aspirating the wash buffer after each wash.

3. Counterstaining and Mounting

3.1. Add the Hoechst (*Ho-kst*) 33342 (*three-three-three-four-two*) nuclear counterstain diluted in TBS, and incubate for 15 minutes at room temperature while protecting the samples from light [1]. Wash the cells three times with TBST, aspirating the wash buffer after each wash [2]. Finally, add 0.5 milliliters of TBS to each well to keep the coverslips submerged until mounting [3].

- 3.1.1. Talent adding the counterstain in the wells and keeping the plate aside in the dark
- 3.1.2. Talent washing the cells three times with TBST and aspirating the wash buffer after each wash
- 3.1.3. Talent adding 0.5 mL of TBS to each well

3.2. While keeping the coverslips submerged in TBS, insert the tip of a fine needle under the edge of the coverslip [1] and gently lift it upward until one side is partially lifted [2]. Grasp the coverslip with fine forceps [3] and carefully remove it from the well [4].

- 3.2.1. Talent inserting the tip of a fine needle under the coverslip.
- 3.2.2. Talent gently lifting the coverslip upward
- 3.2.3. Talent grasping the coverslip with the fine forceps
- 3.2.4. Talent carefully removing the coverslip from the well

3.3. While holding the coverslip with fine forceps, remove excess TBS from the edges using a vacuum aspirator, avoiding contact with the cell-covered surface [1]. Place the coverslip, cell-side down, onto a clean glass slide containing approximately 3 microliters of mounting medium and allow the slides to cure horizontally for 24 hours, protected from light [2]. Place the slide on the fluorescence microscope stage [3] and acquire images [4].

- 3.3.1. Talent removing the excess TBS using vacuum aspirator
- 3.3.2. Talent placing the coverslip onto a glass slide and keeping the slide aside
- 3.3.3. Talent placing the slide on the microscope stage
- 3.3.4. Talent acquiring images

Results

4. Results

4.1. Following the permeabilization with 0.3% non-ionic detergent, p14ARF (*P-fourteen-arf*) shows prominent nuclear staining [1]. In contrast, cells processed without detergent permeabilization show markedly reduced nuclear p14ARF staining [2].

4.1.1. LAB MEDIA: Figure 3A *Video Editor: Please highlight the bright spots indicated by the white arrows*

4.1.2. LAB MEDIA: Figure 3B *Video Editor: Please highlight a few cells showing little or no bright yellow signal*

4.2. In the no-antibody control, nuclear staining is visible [1], while negligible fluorescence is observed in the antibody-dependent channels [2]. Similarly, the secondary-only control shows nuclear staining with no signals in the other fluorescence channels [3], indicating low nonspecific binding of the secondary antibody [4].

4.2.1. LAB MEDIA: Supplementary Figure 2A–E *Video Editor: Please highlight the blue-stained nuclei in A and E*

4.2.2. LAB MEDIA: Supplementary Figure 2A–E *Video Editor: Please highlight the dark areas in B–D*

4.2.3. LAB MEDIA: Supplementary Figure 2F–J *Video Editor: Please highlight the blue-stained nuclei in F and J*

4.2.4. LAB MEDIA: Supplementary Figure 2F–J *Video Editor: Please highlight the dark areas in G–I.*

4.3. The p14ARF single-stain control displays a distinct green, fluorescent signal [1], clearly detectable alongside the nuclear staining in the merged image [2]. In comparison, actin is visualized as strong orange fluorescence throughout the cellular structures [3], and this signal is retained in the merged image [4]. The Interferon-beta single-stain control shows fluorescence in the corresponding far-red channel, with the signal also visible in the merged image [5].

4.3.1. LAB MEDIA: Supplementary Figure 2K–O *Video Editor: Please highlight the bright green spots in figure L*

4.3.2. LAB MEDIA: Supplementary Figure 2K–O *Video Editor: Please highlight the bright green spots in the figure image O*

- 4.3.3. LAB MEDIA: Supplementary Figure 2P–T *Video Editor: Please highlight the bright orange cell structures in Figure R*
- 4.3.4. LAB MEDIA: Supplementary Figure 2P–T *Video Editor: Please highlight the bright orange cell structures in Figure T*
- 4.3.5. LAB MEDIA: Supplementary Figure 2U–Y *Video Editor: Please highlight the bright red cell-associated signal in X, followed by the corresponding signal in Y*

Pronunciation guides:

1. Biosafety
Pronunciation link: <https://www.merriam-webster.com/dictionary/biosafety>
IPA: /ˌbaɪ.əʊˈseɪf.ti/
Phonetic Spelling: bye·oh·sayf·tee
2. Coverslip
Pronunciation link: <https://www.merriam-webster.com/dictionary/coverslip>
IPA: /ˈkʌv.əˌslɪp/
Phonetic Spelling: kuh·ver·slip
3. Confluence
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluence>
IPA: /ˈkɑːn.flu.əns/
Phonetic Spelling: kon·floo·uhns
4. Aspirate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspirate>
IPA: /ˈæs.pəˌreɪt/
Phonetic Spelling: as·puh·rayt
5. Paraformaldehyde
Pronunciation link: No confirmed link found
IPA: /ˌpær.əˈfɔːrˈmæl.dəˌhaɪd/
Phonetic Spelling: pair·uh·for·mal·duh·hyde
6. Permeabilization
Pronunciation link: No confirmed link found
IPA: /ˌpɜː.mi.ə.bəˌleɪˈzeɪ.ʃən/
Phonetic Spelling: per·mee·uh·buh·luh·zay·shuhn
7. Non-ionic
Pronunciation link: No confirmed link found
IPA: /ˌnɑːn.aɪˈɑː.nɪk/
Phonetic Spelling: non·eye·on·ik
8. Tween
Pronunciation link: <https://www.merriam-webster.com/dictionary/tween>

IPA: /twi:n/

Phonetic Spelling: tween

9. Antibody

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

IPA: /'æn.tɪ.bɑː.di/

Phonetic Spelling: an·tee·baa·dee

10. Multiplex

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

IPA: /'mʌl.tə.pleks/

Phonetic Spelling: mul·tuh·pleks

11. Immunofluorescence

Pronunciation link: No confirmed link found

IPA: /ɪ.mjuː.noʊ.flʊ'res.əns/

Phonetic Spelling: ih·myoo·noh·floo·res·uhns

12. Unconjugated

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

IPA: /ʌn'kɑː.n.dʒəˌgeɪ.tɪd/

Phonetic Spelling: un·kon·juh·gay·tid

13. Antigen

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

IPA: /'æn.tə.dʒən/

Phonetic Spelling: an·tuh·juhn

14. Microliter

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

IPA: /'maɪ.kroʊ.liː.tər/

Phonetic Spelling: my·kroh·lee·ter

15. Humidified

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

IPA: /hjuː'mɪd.əˌfaɪd/

Phonetic Spelling: hyoo·mid·uh·fyd

16. Bovine

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

IPA: /'boʊ.vaɪn/

Phonetic Spelling: boh·vine

17. Albumin

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

IPA: /æl'bjʊː.mən/

Phonetic Spelling: al·byoo·muhn

18. Counterstain

Pronunciation link: No confirmed link found

IPA: /'kaʊn.təˌsteɪn/

Phonetic Spelling: kown·ter·stayn

19. Aspirator

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

IPA: /'æs.pə.reɪ.tə/

Phonetic Spelling: as·puh·ray·ter

20. Fluorescence

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

IPA: /flʊ'res.əns/

Phonetic Spelling: floo·res·uhns

21. Microscope

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

IPA: /'maɪ.krə'skɒp/

Phonetic Spelling: my·kruh·skohp

22. Permeabilization

Pronunciation link: No confirmed link found

IPA: /pə:.mi.ə.bə.lə'zeɪ.fən/

Phonetic Spelling: per·mee·uh·buh·luh·zay·shuhn

23. Nonspecific

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

IPA: /,nɑ:n.spə'sɪf.ɪk/

Phonetic Spelling: non·spuh·sɪf·ɪk

24. Actin

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

IPA: /'æk.tɪn/

Phonetic Spelling: ak·tɪn

25. Interferon

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

IPA: /,ɪn.tə'fɪr.ɑ:n/

Phonetic Spelling: in·ter·feer·on