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Title: Iterative Bleaching Extends Multiplicity with Use of Staining Automation for Core Facilities

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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**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   0.25 miles apart (2 buildings on the same campus)

Current Protocol Length
Number of Steps: 14
Number of Shots: 27

Introduction

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

1.1. **Anna Tseng:** Our research focuses on characterizing tissue-specific immune responses during viral infection. We're particularly interested in how viruses reshape local microenvironments and the composition of immune infiltrates over time.

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

What significant findings have you established in your field?

1.2. **Anna Tseng:** We've adapted spatial biology approaches for high biocontainment settings—where prolonged fixation is required and fresh tissue analysis is often impossible—making these techniques feasible for studying these pathogens.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 1*

How will your findings advance research in your field?

1.3. **Anna Tseng:** By making IBEX compatible with autostainers, we hope to increase access to multiplexed tissue staining and allow more labs to explore complex immune responses in spatial context. *Suggested B roll: 2.3*

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

Protocol

2. IBEX Adaptation with Autostainers Using TSA Reagents and Whole Slide Imaging Platforms

Demonstrator: Anna Tseng

2.1. To begin, use a microtome to prepare formalin-fixed paraffin-embedded tissue sections on positively charged slides **[1-TXT]**. Let them dry at room temperature for 10-15 minutes **[2],** then bake at 60 degrees Celsius overnight **[3]**.

2.1.1. WIDE: Talent operating a microtome to cut sections. **TXT: One slide/sample, +ve controls, and a PBS-treated -ve control**

2.1.2. Talent placing slides on a drying rack at room temperature.

2.1.3. Move slides to oven set at 60^0C.

2.2. Prepare all antibodies and dyes required for the first imaging panel of the IBEX *(I-Bex)* protocol **[1]**. Then, set up the autostaining program with the selected panel configuration **[2-TXT]**.

2.2.1. Shot of labeled antibody vials and fluorescent dyes.

2.2.2. Display the autostainer interface showing selection of a 5-marker panel. **TXT: Ensure three washing steps between each reagent application**

2.3. Next, load the tissue slides and antibody solutions into the autostainer before running the automated staining program **[1]**. NOTE: The VO is edited for the deleted shot

2.3.1. Talent loading slides into the autostainer tray and inserting antibody reservoirs.

~~2.3.2. Talent pressing **Start** on the touchscreen.~~ NOTE: Not filmed

2.4. Then, remove the slides from the autostainer **[1]** and submerge them in wash solution **[2].** Now, using aqueous mounting media suitable for multispectral imaging, apply a number 1.5 coverslip **[3-TXT]**.

2.4.1. Talent removing stained slides from the autostainer.

2.4.2. Talent dunks slides in wash solution.

2.4.3. Talent applying mounting media and a coverslip. **TXT: Image the slide within 2 days**

2.5. For fluorescence Imaging on epifluorescence, load the well-controlled positive control

sample into the fluorescence slide scanner **[1]**. Locate a positive signal in the sample for each antigen that will be imaged **[2]**. For each fluorescent dye, set the exposure time by starting low and gradually increasing until a signal is observed, ensuring dynamic range capture and minimal background **[3]**.

2.5.1. Talent inserting the positive control slide into the fluorescence slide scanner.

2.5.2. SCREEN: Talent navigates to locate positive signals corresponding to different antigens. NOTE: Insert missing media cards for 2.5.2, 2.5.3 and 2.6.1

2.5.3. SCREEN: Show the interface adjusting the exposure slider for each dye channel, starting low and increasing until the signal appears clearly.

2.6. Then, save the exposure settings determined using the positive control sample for use in all subsequent imaging steps **[1-TXT]**.

2.6.1. SCREEN: Show user saving the exposure settings within the imaging software, labeling it as "Positive Control Profile settings." **TXT: Image all samples using +ve control settings; Confirm image quality before proceeding**

2.7. For coverslip removal, place the slides in a Coplin jar filled with deionized water **[1]** and immerse the jar in a 65 degrees Celsius water bath for 15 to 20 minutes to naturally remove the coverslip **[2]**.

2.7.1. Talent placing slides into a Coplin jar.

2.7.2. Talent placing the jar in a 65 degrees Celsius water bath.

2.8. To bleach the sections, freshly prepare a 1 milligram per milliliter solution of lithium borohydride in deionized water inside a fume hood **[1-TXT]**. Loosely cover the tube and allow the lithium borohydride solution to rest for 20 minutes to enable bubble formation **[2]**.

2.8.1. Talent pipetting lithium borohydride powder into a beaker on a balance inside the fume hood. **TXT: Make enough solution to cover each sample 3x over**

2.8.2. Talent loosely covers tube with solution and lets it rest until bubbles form. NOTE: Labeled as 2.8.1B in shot, but actually is 2.8.2

2.9. Then, pipette 1 milliliter of lithium borohydride solution onto each slide placed in a staining chamber inside the fume hood and allow it to sit for 10 to 15 minutes **[1-TXT]**.

2.9.1. Talent dispensing 1 milliliter of lithium borohydride onto each slide and closing the chamber for incubation. **TXT: Replace $LiBH_4$ solution with fresh solution and**

repeat 2x

2.10. After removing the final lithium borohydride treatment from the slides, transfer the slides into PBS or deionized water for short-term storage of no more than 1 day **[1-TXT]**.

2.10.1. Talent transferring slides from the staining chamber to a fresh Coplin jar containing phosphate-buffered saline or deionized water. **TXT: Proceed to next IBEX round within 1 day of bleaching**

2.11. Then, prepare all antibodies and tyramide signal amplification dye solutions required for the next round of autostaining **[1]**.

2.11.1. Shot of all antibodies and tyramide signal amplification dye solutions.

2.12. For the Ventana autostainer, load the prepared slides into the autostainer tray **[1].** Then, pipette approximately 1 milliliter of autostainer wash buffer onto the slide to maintain the wet mount before starting the autostaining run **[2]**.

2.12.1. Talent loading prepared slides into the Ventana autostainer tray. NOTE: 2.12.1 and 2.12.2 are filmed in a single shot

2.12.2. Talent pipetting wash buffer directly onto a slide inside a humidity-controlled environment.

2.13. Now, set up the autostainer program according to the panel configuration, with the number of cycles set to match the number of antibodies in the staining round **[1]**.

2.13.1. Display the Ventana programming interface where the user selects a panel and sets the parameter **N** to the number of antibodies to be applied.

2.14. Afterward, remove the slides from the autostainer **[1]** and apply a number 1.5 coverslip using an aqueous mounting medium compatible with multispectral imaging **[2]**. Then, image the stained slides within 2 days using a positive control sample to set the imaging parameters for the new set of markers **[3-TXT]**.

2.14.1. Talent removing slides from the autostainer.

2.14.2. Talent apply coverslips with a drop of mounting medium.

2.14.3. Talent loading freshly stained slides onto a fluorescence imaging microscope and starting image capture. **TXT: After imaging, bleach the slides and repeat for all IBEX panels desired**

Results

3. Results

3.1. The spatial organization of immune cells, inflammatory markers, and specialized lung cells was visible in lung tissue infected with *Mycobacterium tuberculosis* **[1].**

3.1.1. LAB MEDIA: Figure 4.

3.2. Optimization of TSA reagents allowed markers to appear with minimal background staining **[1].**

3.2.1. LAB MEDIA: Figure 4. *Video editor: Pan across individual marker panels (e.g., iNOS, CD11b, CD206, CD163) to show clearly visible markers with dark, clean backgrounds.*

1. **Microtome**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/microtome
   - IPA: /ˈmaɪkroʊˌtoʊm/
   - Phonetic spelling: MY-kroh-tohm
2. **Formalin**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/formalin
   - IPA: /ˈfɔːrməlɪn/
   - Phonetic spelling: FOR-muh-lin
3. **Paraffin**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/paraffin
   - IPA: /ˈpærəfɪn/
   - Phonetic spelling: PAIR-uh-fin
4. **IBEX** *(I-Bex)*
   - Pronunciation link: No confirmed link found
   - IPA: /ˈaɪˌbɛks/
   - Phonetic spelling: EYE-bex

5. **Tyramide**
    - Pronunciation link: https://www.howtopronounce.com/tyramide
    - IPA: /ˈtɪrəˌmaɪd/
    - Phonetic spelling: TIR-uh-myd
6. **Epifluorescence**
    - Pronunciation link (for "fluorescence"): https://www.merriam-webster.com/dictionary/fluorescence
    - IPA: /ˌɛpiˌflʊˈrɛsəns/
    - Phonetic spelling: EH-pee-fluh-RESS-ens