Submission ID #: 67980
Scriptwriter Name: Poornima G
Project Page Link: https://review.jove.com/account/file-uploader?src=20733948

Title: Optimized Workflow for Iterative Bleaching Extends Multiplexity Imaging of Highly Autofluorescent Clinical Samples

Authors and Affiliations:

Aleksandra Lunich[1], Andrea J. Radtke[2,3], Margaret Williams[1], Julia M. Stern[1], Daniel L. Barber[4], Ronald N. Germain[2], Ifeanyichukwu U. Anidi[1,4]

[1]Critical Care Medicine and Pulmonary Branch, National Heart, Lung and Blood Institute, National Institutes of Health
[2]Lymphocyte Biology Section and Center for Advanced Tissue Imaging, Laboratory of Immune System Biology, NIAID, NIH
[3]Leica Microsystems Inc.
[4]T Lymphocyte Biology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health

Corresponding Authors:
Ifeanyichukwu U. Anidi Ifeanyichukwu.anidi@nih.gov; Ifeanyi.anidi@gmail.com

Email Addresses for All Authors:
Aleksandra Lunich ally.lunich@icloud.com
Andrea J. Radtke andrea.radtke@leica-microsystems.com
Margaret Williams margowilliams027@gmail.com
Julia M. Stern jstern925@gmail.com
Daniel L. Barber barberd@niaid.nih.gov
Ronald N. Germain rgermain@niaid.nih.gov
Ifeanyichukwu U. Anidi Ifeanyichukwu.anidi@nih.gov; Ifeanyi.anidi@gmail.com

Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

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

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **MM/DD/YYYY**
Authors, please provide a date why when you can complete the interviews

When you are ready to submit your video files, please contact our Content Manager, Utkarsh Khare.

Current Protocol Length

Number of Steps: 16
Number of Shots: 29

Introduction

@VO team, please record the Introduction statements also.

What is the scope of the research? NOTE to editor: Please include the first question.

1.1. Using spatial proteomics, named Nature Methods' 2024 *(Twenty-Twenty Four)* Method of the Year, immune cells' precise locations can be mapped and interactions within tissues. This reveals spatial patterns linked to clinical outcomes.

1.1.1. *2.3.1*

What are the current experimental challenges?

1.2. Building complex antibody panels and imaging tissues with high-endogenous fluorescence presents significant time, resources, and expertise challenges for IBEX and other fluorescence microscopy techniques.

1.2.1. *2.6.1*

What significant findings are established in the field?

1.3. Using IBEX *(i-bex)*, tumor-specific features were identified in high-risk follicular lymphoma patients, and with Human Cell Atlas, a comprehensive spatial map of the human thymus was created.

1.3.1. *2.9.1*

What research gaps are addressed with this protocol?

1.4. Little is known about the immune cell composition, spatial interactions, and cytokine production differences across diverse mycobacterial pulmonary pathologies. This protocol bridges this gap.

1.4.1. *3.4.2*

What advantage does this protocol offer compared to other techniques?

1.5. This protocol offers a low-cost, adaptable photoirradiation method to significantly reduce broad-spectrum tissue autofluorescence, especially in difficult FFPE *(F-F-P-E)* samples, while preserving antibody signal for spatial analysis.

1.5.1. *4.2.1*

Ethics Title Card

This research has been approved by the Institutional Review Board (IRB) at National Institutes of Health

Protocol

2. Antibody Labeling Prior to Photoirradiation

Demonstrator: Aleksandra Lunich

2.1. To begin, immerse the slides with tissue in PBS after completing antigen retrieval **[1]**.

2.1.1. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 00:05-00:10.

2.2. Remove one slide from the PBS **[1]** and use a lint-free wipe to carefully dry off all excess buffer without touching the tissue **[2]**. With a hydrophobic pen, draw a border around the tissue section on the slide **[3]**. After repeating the process for the remaining slides, let the hydrophobic barriers dry for 10 minutes **[4]**.

2.2.1. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 00:11-00:17.

2.2.2. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 00:50-00:55.

2.2.3. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 01:08-01:15.

2.2.4. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 02:25-02:35.

2.3. Then, using a lint-free wipe, wick off the PBS from the tissue section without touching the tissue directly **[1]**.

2.3.1. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 03:45-03:55.

2.4. Now, add 200 microliters of blocking buffer on the slide **[1]** and close the moisture chamber **[2]**.

2.4.1. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 04:00-04:10.

2.4.2. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 04:15-04:22.

2.5. Place the chamber in a non-heating scientific microwave with a temperature-regulating

mechanism and run the previously established program 5 cycles **[1-TXT]**.

2.5.1. IBEX_03_Drawing Hydrophobic Barrier on Slide and Setting Up For Blocking Timestamps: 04:28-04:35. **TXT: Program cycle: 2 min at 100 W and 1 min at 0 W**

2.6. During the blocking incubation, prepare the primary antibody staining solution 1 with Hoechst and the blocking buffer containing Fc Block **[1]**. Combine the antibodies and gently mix the cocktail **[2]**.

2.6.1. IBEX_04_Creating and Adding First Primary Antibody Mixture Timestamps: 00:18-00:25.

2.6.2. IBEX_04_Creating and Adding First Primary Antibody Mixture Timestamps: 00:40-00:48.

2.7. When the microwave cycle completes, remove and open the slide chamber **[1]** and wick off the blocking buffer from the slide without touching the tissue **[2]**.

2.7.1. IBEX_04_Creating and Adding First Primary Antibody Mixture Timestamps: 00:00-00:08.

2.7.2. IBEX_04_Creating and Adding First Primary Antibody Mixture Timestamps: 01:15-01:25.

2.8. Next, add 200 microliters of primary antibody staining solution 1 to the slide **[1]**. Return the chamber to the microwave and execute the primary antibody program again for approximately 30 minutes **[2]**.

2.8.1. IBEX_04_Creating and Adding First Primary Antibody Mixture Timestamps: 01:45-01:55.

2.8.2. IBEX_04_Creating and Adding First Primary Antibody Mixture Timestamps: 02:10-02:19.

2.9. Once the primary antibody labeling is complete, hold the slide vertically **[1].** To wash the slide, pipette 1,000 microliters of PBS onto the tissue, allowing it to run off **[2-TXT]**.

2.9.1. IBEX_05_Washing Off Primary Antibodies and Adding PFA For Fixation Timestamps: 00:20-00:30.

2.9.2. IBEX_05_Washing Off Primary Antibodies and Adding PFA For Fixation Timestamps: 00:30-00:40. **TXT: Repeat the washing step 3 - 5x**

2.10. Wick off the excess PBS **[1]** and fix the slide with 1 percent paraformaldehyde for 10

minutes at room temperature in the humidity chamber **[2]**.

2.10.1. IBEX_05_Washing Off Primary Antibodies and Adding PFA For Fixation Timestamps: 01:20-01:25.

2.10.2. IBEX_05_Washing Off Primary Antibodies and Adding PFA For Fixation Timestamps: 02:10-02:25.

2.11. After fixing, wash the slide thoroughly with 1,000 microliters of PBS three to five times and leave a layer of PBS on the tissue until the photoirradiation step **[1]**.

2.11.1. IBEX_06_Placing Slides In PBS After Fixation and Before Photobleaching Timestamps: 00:00-00:11.

3. Tissue Photoirradiation Procedure

3.1. To prepare the photoirradiation box, gather a 150-watt LED *(L-E-D)* lamp, a 40-watt RGBW flood LED lamp **[1]**, a large plastic container with a capacity of 75.71 liters or more, fresh PBS, and a Petri dish **[2]**.

3.1.1. IBEX_07_Setting Up Slides in Photobleaching Box with Explanation Timestamps: 00:06-00:12.

3.1.2. IBEX_07_Setting Up Slides in Photobleaching Box with Explanation Timestamps: 00:25-00:30.

3.2. Now, fill the Petri dish with 1× PBS to completely submerge the slide **[1]**. Carry out the photoirradiation procedure in a cold room to minimize heat from the lamps **[2]**.

3.2.1. IBEX_07_Setting Up Slides in Photobleaching Box with Explanation Timestamps: 00:52-01:00.

3.2.2. IBEX_07_Setting Up Slides in Photobleaching Box with Explanation Timestamps: 01:00-01:05.

3.3. Then, place the slide in the Petri dish, ensuring it is fully submerged in PBS **[1]**.

3.3.1. IBEX_07_Setting Up Slides in Photobleaching Box with Explanation Timestamps: 01:15-01:25.

3.4. Next, place the 40-Watt lamp directly above the Petri dish with the light source facing downward toward the slide **[1]** and set the lamp to the red-light mode **[2]**.

3.4.1. IBEX_07_Setting Up Slides in Photobleaching Box with Explanation Timestamps: 01:26-01:38.

3.4.2. IBEX_07_Setting Up Slides in Photobleaching Box with Explanation Timestamps: 01:44-01:46.

3.5. Finally, turn on both the 150-watt and 40-watt lamps **[1]** and cover the entire setup with the plastic container lid. After 2 hours, switch the lamp to green light and incubate for 16 hours **[2-TXT]**.

3.5.1. IBEX_08_Red Setting Photobleaching Box Timestamps: 00:00-00-05.

3.5.2. IBEX_08_Red Setting Photobleaching Box Timestamps: 00:06-00-15 **TXT: Stain with Ab post-irradiation; Perform microscopic assessment**

Results

4. Results

4.1. The brightest fluorophores compatible with the dye inactivation protocol were identified and paired with lowly expressed markers to enhance signal detection **[1]**. Autofluorescence was reduced significantly after photoirradiation **[2]** and at longer imaging wavelengths **[3]**.

4.1.1. LAB MEDIA: Figure 3A

4.1.2. LAB MEDIA: Figure 3A. *Video editor: Highlight the row "+Photoirradiation*".

4.1.3. LAB MEDIA: Figure 3B. *Video editor: Focus on the lower rows labeled C1 and C2*.

4.2. Directly conjugated antibodies targeting highly expressed structural markers, alpha-smooth muscle actin **[1]** and pan-cytokeratin, were placed in the near-infrared channel at 750 nanometers **[2]**.

4.2.1. LAB MEDIA: Figure 3B. *Video editor: Zoom in on image "C1 α-SMA", the last image in row 2 labeled "C1".*

4.2.2. LAB MEDIA: Figure 3B. *Video editor: Zoom in on image "C2 PanCK", the last image in row 3 labeled C2*.

4.3. Background signal was computationally subtracted using an unstained reference image and SimpleITK *(Simple-I-T-K)* arithmetic **[1]**, and true signals were enhanced through thresholding **[2]**.

4.3.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the "corrected" image*.

4.3.2. LAB MEDIA: Figure 3D. *Video editor: Highlight the "thresholded" image*.

4.4. Whole-slide imaging of IBEX *(i-bex)*-stained sections revealed granulomas with necrotic cores and CD15 *(C-D-Fifteen)*-positive neutrophils **[1]**.

4.4.1. LAB MEDIA: Figure 4, Inset 3. *Video editor: Zoom in on MERGE and CD15 images*

4.5. *Mycobacterium tuberculosis* or nontuberculous mycobacteria were detected near the granuloma necrotic core using anti-Ag85B *(A-G-Eighty-Five-B)* antibody **[1]**.

4.5.1. LAB MEDIA: Figure 4, Inset 3. *Video editor: Zoom in on Ag85B image*.

4.6. Granuloma-associated lymphoid tissue contained CD20 *(C-D-Twenty)*-positive B cells

[1], CD4 *(C-D-Four)*-positive T cells **[2]**, a few CD8 *(C-D-Eight)*-positive T cells **[3]**, and lumican expression indicating extracellular matrix **[4]**.

4.6.1. LAB MEDIA: Figure 4, Inset 5. *Video editor: highlight the image CD20*

4.6.2. LAB MEDIA: Figure 4, Inset 5. *Video editor: highlight the images CD4*

4.6.3. LAB MEDIA: Figure 4, Inset 5. *Video editor: highlight the image CD8*

4.6.4. LAB MEDIA: Figure 4, Inset 5. *Video editor: highlight the image CD45*.

4.7. IBEX imaging also enabled visualization of lung anatomical features, including bronchial epithelial cells, arterial smooth muscle, and CD68 *(C-D-Sixty-Eight)*-positive macrophages **[1]**.

4.7.1. LAB MEDIA: Figure 4, Inset 2. *Video editor: Mark the regions labeled as epithelium, artery, and macrophages*.

Pronunciation guide:

1. hydrophobic

Pronunciation link:
https://www.merriam-webster.com/dictionary/hydrophobic
howtopronounce.com+5dictionary.cambridge.org+5merriam-webster.com+5youtube.com+11merriam-webster.com+11merriam-webster.com+11
IPA: /ˌhaɪdroʊˈfoʊbɪk/
Phonetic spelling: hy-droh-FOH-bik

---

2. photoirradiation

Pronunciation link:
https://www.howtopronounce.com/photoirradiation
en.wiktionary.org+2howtopronounce.com+2howtopronounce.com+2
IPA: /ˌfoʊtoʊˌɪreɪdiˈeɪʃən/
Phonetic spelling: fo-toh-ih-ray-dee-AY-shuhn

---

3. paraformaldehyde

No confirmed Merriam-Webster link found (check "no confirmed").
IPA: /ˌpærəˌfɔːr.məlˈdiːhaɪd/
Phonetic spelling: par-uh-for-mal-DEE-hyde

---

4. autofluorescence

No confirmed Merriam-Webster link found.
IPA: /ˌɔːtoʊˌflʊəˈrɛsəns/
Phonetic spelling: AW-toh-flu-oh-RESS-uhns

---

5. granuloma

No confirmed Merriam-Webster link found.
IPA: /ˌgrænjəˈloʊmə/
Phonetic spelling: gran-yoo-LOH-muh

---

6. necrotic

No confirmed Merriam-Webster link found.
IPA: /nɛˈkrɒtɪk/
Phonetic spelling: neh-KROT-ik

7. lumican

No confirmed Merriam-Webster link found.
IPA: /ˈluːmɪkæn/
Phonetic spelling: LOO-mi-kan

8. Mycobacterium tuberculosis

No confirmed Merriam-Webster link found.
IPA: /ˌmaɪkoʊbækˈtɪəriəm tubərkjuˈloʊsɪs/
Phonetic spelling: my-coe-back-TIAR-ee-um too-ber-kyuh-LOH-sis

9. nontuberculous

No confirmed Merriam-Webster link found.
IPA: /ˌnɑːn.tuːˈbɜːrkjələs/
Phonetic spelling: non-too-BURK-yuh-lus

10. cytokeratin

IPA (American): /ˌsaɪˌtoʊˈkɛrətɪn/

Phonetic Spelling: sy-toh-KER-uh-tin