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Title: High-Plex Imaging Using Spectral Confocal Microscopy to Minimize Non-Specific Tissue Fluorescence

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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**

   If **Yes**, how far apart are the locations? **500 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. **Yes**

Current Protocol Length
Number of Steps: 25
Number of Shots: 57 (32 SC)

Introduction

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

INTRODUCTION:

1.1. **Vitoria Olyntho:** We optimized an IBEX workflow combining heparin blocking and spectral detection to suppress background in eosinophil-rich human tissues.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

1.2. **Vitoria Olyntho:** Autofluorescence and eosinophil granule binding obscure true signal; we target both to enable clean, high-plex maps

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

CONCLUSION:

1.3. **Saven Denha:** Heparin treatment eliminated granule binding without reducing true signal, maintaining strong background suppression across IBEX cycles.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.1*

1.4. **Saven Denha:** Spectral unmixing minimized bleed-through and autofluorescence, increasing signal-to-background and significantly speeding acquisition versus multi-track confocal.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.3.1*

1.5. **Dima Traboulsi:** This enables sensitive, robust spatial proteomics in challenging tissues, supporting accurate cell-state mapping and neighborhood-level analysis.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.1.2*

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

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Dima Traboulsi**: (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.1*

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Saven Denha, PhD candidate**: (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.3.1*

Ethics Title Card

The use of nasal polyps was approved by the Hamilton Integrated Research Ethics Board

Protocol

2. Blocking the Mounted Sample and Antibody Immunolabeling

Demonstrator: Dima Traboulsi

2.1. To begin, draw a circle on the glass slide around the mounted tissue using a PAP pen to create a hydrophobic barrier **[1-TXT]**.

2.1.1. **WIDE:** Talent drawing a circle around the tissue section on a glass slide using a PAP pen. **TXT: Avoid drawing over the optimal cutting temperature compound area**

Videographer's NOTE: 2.1.1 – take 1 wide shot, take 2-4 close up

2.2. Add approximately 100 microliters of PBS to each tissue to rehydrate them **[1]** and incubate the slide for about 2 minutes at room temperature to wash off the optimal cutting temperature compound **[2]**.

2.2.1. Talent pipetting 100 microliters of PBS onto the tissue within the PAP pen boundary. **Authors**: I am omitting the information regarding the chambered cover glasses at every step as we are already over the limit of 55 shots. Please use normal cover glasses for filming as the volumes of buffers stated here are as per those **Videographer's NOTE:** 2.2.1 – shot in 4K

2.2.2. Talent placing the slide aside.

2.3. Aspirate the PBS from around the tissue using a pipette **[1]**. Then, add 100 microliters of blocking buffer to each tissue **[2]** and incubate at room temperature in a covered humidity chamber for 1 hour **[3]**.

2.3.1. Talent aspirating PBS from the glass slide. **Videographer's NOTE**: 2.3.1 and 2.3.2 – shot in 4K

2.3.2. Talent pipetting 100 microliters of blocking buffer into the hydrophobic circle.

2.3.3. Talent placing the slide into a covered humidity chamber at room temperature.

2.4. Now, aspirate the blocking buffer using a pipette **[1]**. Add 100 microliters of staining mix to each tissue **[2]** and incubate the slide in a covered humidity chamber inside a refrigerator at 4 degrees Celsius overnight **[3].**

2.4.1. Talent aspirating blocking buffer from the glass slide.

Videographer's NOTE: 2.4.1 and 2.4.2 – shot in 4K

2.4.2. Talent pipetting 100 microliters of staining mix onto the tissue within the PAP pen boundary.

2.4.3. Talent placing the covered humidity chamber inside a refrigerator set at 4 degrees Celsius.

2.5. Prepare sections stained with only one fluorophore and a negative control without any stain to define the fluorophore spectrum and tissue autofluorescence **[1]**.

2.5.1. Talent labeling additional slides for single fluorophore staining and negative control.

2.6. After aspirating the staining mix, wash the tissue three times with PBS **[1-TXT]**. Aspirate the liquid after each wash **[2]**. Mount the sample on the microscope for imaging **[3-TXT]**.

2.6.1. Talent pipetting PBS onto the tissue. **TXT: Add 100 µL PBS; Incubate for 2 min between washes**

Videographer's NOTE: 2.6.1 and 2.6.2 – shot in 4K

2.6.2. Talent aspirating liquid between washes.

Videographer's NOTE: 2.6.2 – take 2 is mounting

2.6.3. Talent placing the sample on the microscope stage. **TXT: Do not use the DAPI filter when focusing the sample using the eyepieces**

Videographer's NOTE: 2.6.3 – take 1 wide shot, take 2 close up

3. Spectral Confocal Microscopy

Demonstrator: Saven Denha

3.1. Focus on the sample using brightfield or differential interference contrast mode **[1]**. Then switch to fluorescence mode and configure the spectral acquisition parameters according to the experimental requirements **[2]**.

3.1.1. SCREEN: 68644_screenshot_1.mp4 00:15-00:31

3.1.2. SCREEN: 68644_screenshot_2.mp4.

3.2. Assign the appropriate lasers for the fluorophores used in the experiment **[1]**.

3.2.1. SCREEN: 68644_screenshot_3.mp4.

3.3. Select a beam splitter that corresponds to the chosen laser lines **[1]**. Enable all detectors to capture the full emission spectrum **[2]** and adjust the pinhole size to 1 Airy Unit for optimal optical sectioning **[3]**.

3.3.1. SCREEN: 68644_screenshot_4.mp4.

3.3.2. SCREEN: 68644_screenshot_5.mp4.

3.3.3. SCREEN: 68644_screenshot_6.mp4.

3.4. Optimize the laser settings before acquisition. Using a section stained with all markers, start **Continuous scanning** mode at low laser power **[1]**. Gradually increase laser intensity until all markers are identifiable with a good signal-to-background ratio **[2]**.

3.4.1. SCREEN: 68644_screenshot_7.mp4.

3.4.2. SCREEN: 68644_screenshot_8.mp4 0:10-00:15, 01:35-01:40.

3.5. Fine-tune laser intensities and iteratively alternate between single-stain and multi-stain sections until all fluorophore signals are visible without oversaturation **[1]**.

3.5.1. SCREEN: 68644_screenshot_9.mp4 00:00-00:20.

3.6. Enable the **Tiles** feature in the acquisition software **[1]** and define the tile acquisition region around the tissue to ensure full image coverage **[2]**.

3.6.1. SCREEN: 68644_screenshot_10.mp4.

3.6.2. SCREEN: 68644_screenshot_11.mp4.

3.7. Optimize the focus for the tile acquisition region at the center of the tissue **[1]**. Identify a unique structural feature, such as a distinct set of cells in a recognizable arrangement, to serve as a reference for alignment in later imaging rounds **[2]**.

3.7.1. SCREEN: 68644_screenshot_12.mp4.

3.7.2. SCREEN: 68644_screenshot_13.mp4. 00:05-00:25

3.8. Now, enable the **Z-stack** feature and navigate to the **Z-stack** tab **[1]**. Under the **Center** tab, set the number of slices to at least 6, with an interval of no less than 1 micrometer between slices **[2]**. Click the **Center** button to set the current focus point, determined in the previous step, as the middle slice of the stack **[3]**.

3.8.1. SCREEN: 68644_screenshot_14.mp4.

3.8.2. SCREEN: 68644_screenshot_15.mp4.

3.8.3. SCREEN: 68644_screenshot_16.mp4.

3.9. Click the **Start experiment** button to initiate image acquisition **[1]**. Once acquisition is complete, right-click on the image thumbnail, select **Save As**, choose a file location, and name the image **[2]**. Backup the image data to secure storage **[3]**.

3.9.1. SCREEN: 68644_screenshot_17.mp4.

3.9.2. SCREEN: 68644_screenshot_18.mp4.

3.9.3. SCREEN: 68644_screenshot_19.mp4.

3.10. Unmix the image using the same optimized settings applied during tiled region acquisition **[1]**. Capture a **Snap** from each single-stained control to obtain the true signal spectrum and from the unstained control to capture background or autofluorescence **[2]**.

3.10.1. SCREEN: 68644_screenshot_20.mp4 00:00-00:20.

3.10.2. SCREEN: 68644_screenshot_21.mp4 00:03-00:15.

3.11. Use these images exclusively for spectral unmixing. Confirm that the selected region represents a true signal by checking that the intensity and morphology are absent or equivalent to background levels in the unstained control **[1]**, and that the marker's localization matches its expected cellular pattern, such as membrane, nuclear, cytoplasmic, or secreted distribution **[2]**.

3.11.1. SCREEN: 68644_screenshot_22.mp4.

3.11.2. SCREEN: 68644_screenshot_23.mp4.

3.12. Open the snap image in the software **[1]**. Click on the **Unmixing** tab and select one of the region selection tools under the **Unmix tools** tab **[2]**. Draw a region of interest (ROI) around the positive signal to generate the spectrum of that region **[3-TXT]**.

3.12.1. SCREEN: 68644_screenshot_24.mp4.

3.12.2. SCREEN: 68644_screenshot_25.mp4.

3.12.3. SCREEN: 68644_screenshot_26.mp4. **TXT: Repeat this process for each fluorophore to create reference spectra**

3.13. Compare each generated spectrum to the theoretical emission spectrum of the

corresponding fluorophore **[1]**. Ensure that the region accurately represents the true signal, name each spectrum according to the fluorophore identity, and save it to the spectral database **[2]**.

3.13.1. SCREEN: 68644_screenshot_27.mp4.

3.13.2. SCREEN: 68644_screenshot_28.mp4 00:15-00:25.

3.14. Next, load the tiled image and open the **Unmixing** tool **[1]**. Use the **+** *(plus)* button to add the reference spectra for each fluorophore to the unmixing list **[2]**. Click the **Linear unmixing** button to separate the spectral signals and save the unmixed image **[3]**.

3.14.1. SCREEN: 68644_screenshot_29.mp4.

3.14.2. SCREEN: 68644_screenshot_30.mp4. 00:00-00:20

3.14.3. SCREEN: 68644_screenshot_31.mp4.

4. Fluorophore Bleaching and Cycling

4.1. Prepare the bleaching solution in a fume hood **[1]**. Using a micro spatula, weigh 10 milligrams of lithium borohydride directly into a dry beaker **[2]**. Carefully add 10 milliliters of ultrapure water into the beaker and mix until the compound is fully dissolved **[3-TXT]**.

4.1.1. Talent placing the containers in the fume hood, wearing proper protective equipment.

Videographer's NOTE: 4.1.1 – shot in 4K, take 1 wide shot, take 2 close up

4.1.2. Talent measuring 10 milligrams of lithium borohydride into a dry beaker using a micro spatula.

4.1.3. Talent adding 10 milliliters of ultrapure water into the beaker and stirring until dissolved. **TXT: Exercise extreme caution as $LiBH_4$ is highly flammable**

4.2. Add approximately 100 microliters of the freshly prepared bleaching solution to each tissue section using a glass Pasteur pipette **[1]** and incubate the slides at room temperature for 15 minutes inside the fume hood **[2]**.

4.2.1. Talent pipetting bleaching solution onto tissue sections within the fume hood.

Videographer's NOTE: 4.2.1 and 4.2.2 – shot in 4K

4.2.2. Talent placing the slide on the fume hood's work surface.

4.3. If bleaching Brilliant Violet dyes such as BV-421 or BV-510, carefully transfer the slide to an epifluorescent microscope **[1]** and select the **DAPI filter**, focus the sample, and illuminate it at full power **[2]**.

4.3.1. Talent transferring the slide from the fume hood to the microscope stage.

4.3.2. SCREEN: 68644_screenshot_31.mp4.

4.4. Now, use a 5X objective to achieve a larger field of illumination **[1]** and lower the stage completely to prevent any contact between the slide and the objective or housing **[2]**.

4.4.1. Show the 5× objective being selected.

Videographer's NOTE: 4.4.1 – combined with 4.4.2

4.4.2. Talent lowering the stage and ensuring safe clearance between the sample and the objective.

4.5. Finally, decant the lithium borohydride solution from each well using a glass Pasteur pipette and return it to the original beaker of bleaching solution **[1]**. Wash the slides three times with dilution buffer and incubate for approximately 3 minutes during each wash **[2]**. Add the antibody mix and stain the sample as demonstrated earlier **[3]**.

4.5.1. Talent aspirating the bleaching solution from the slide and transferring it back into the beaker.

4.5.2. Talent pipetting dilution buffer onto the slide for the first wash.

4.5.3. ~~Talent placing the slide aside for incubation~~. **Videographer's NOTE:** 4.5.3 – shot removed, VO adjusted

4.5.4. Talent adding the antibody mix to the sample.

Results

5. Results

5.1. In untreated nasal polyp tissue, staining with CD3, CD4, and CD20 showed intense, non-specific granular fluorescence in eosinophil-rich regions **[1]**, while true membrane-localized signal was present at lower intensity **[2]**.

5.1.1. LAB MEDIA: Figure 1A. *Video editor: Highlight the yellow arrows pointing to the cells with bright, granular fluorescence in the CD3, CD4, and CD20 panels*.

5.1.2. LAB MEDIA: Figure 1A. *Video editor: Highlight the blue arrows pointing to the dimmer, membrane-localized signals for CD3 and CD4*.

5.2. Pretreatment with 5% skim milk failed to block non-specific fluorescence and also reduced signal intensity for fluorophores such as AF532 **[1]**.

5.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the CD45 (AF532) panel showing reduced signal intensity compared to untreated tissue*.

5.3. DAB pretreatment reduced non-specific fluorescence from CD20, CD3, and CD4 **[1-TXT]**, but also decreased true signal intensity across multiple channels including DAPI **[2]**.

5.3.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the CD20, CD3, and CD4 panels showing reduced granular signa*l. **TXT: DAB: 3,3'-Diaminobenzidine**

5.3.2. LAB MEDIA: Figure 1C. *Video editor: Highlight the DAPI panel showing visibly reduced nuclear staining*.

5.4. Pretreatment with heparin effectively blocked non-specific binding while maintaining high signal intensity for CD3, CD4, CD20, and DAPI **[1]**.

5.4.1. LAB MEDIA: Figure 1D. *Video editor: Highlight all panels sequentially*.

5.5. Heparin blocked non-specific signal in a concentration-dependent manner, with complete suppression achieved at 10 units **[1]**, and no detrimental effects observed at higher concentrations **[2]**.

5.5.1. LAB MEDIA: Figure 1E. *Video editor: Highlight the 10 U panel showing strong signal and minimal background*.

5.5.2. LAB MEDIA: Figure 1E. *Video editor: Highlight the 20 U panel to show that signal and tissue integrity remain unaffected*.

5.6. A 3-track imaging setup reduced acquisition time but introduced crosstalk, including bleed-through of AF594 into the PE channel **[1]**, and enhanced tissue autofluorescence due to multiple laser excitations **[2]**.

5.6.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the area inside small white box*

5.6.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the yellow arrows pointing to autofluorescent regions*.

5.7. Spectral imaging eliminated nearly all background and crosstalk, while also reducing acquisition time and improving signal-to-background ratio **[1]**.

5.7.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the area inside small white box.*

5.8. Spectral IBEX *(I-bex)* imaging of a human nasal polyp revealed epithelial structures marked by EpCAM **[1]**, immune cells such as CD3-positive and CD20-positive lymphocytes **[2]**, and vessels expressing CD31 and CD34 **[3]**.

5.8.1. LAB MEDIA: Figure 3. *Video editor: Highlight the image with EpCAM to show epithelial structures*.

5.8.2. LAB MEDIA: Figure 3. *Video editor: Highlight the images showing CD4 CD3 and CD31 CD20 staining.*

5.8.3. LAB MEDIA: Figure 3. *Video editor: Highlight the images CD31 CD20 and HLA-DR CD34.*

- **fluorophore**

Pronunciation link: https://www.howtopronounce.com/fluorophore howtopronounce.com+2Wikipedia+2
IPA: /ˈflʊərəˌfɔr/ or /ˈflʊəroʊˌfɔr/
Phonetic Spelling: FLUR-uh-for

- **autofluorescence**

Pronunciation link: https://www.howtopronounce.com/autofluorescence (if available, otherwise similar pattern combining "auto" + "fluorescence") — at present: No confirmed link found
IPA: /ˌɔːtoʊˌflʊəˈrɛsəns/
Phonetic Spelling: aw-toh-flu-uh-RESS-ens

- **diaminobenzidine**

Pronunciation link: https://youglish.com/pronounce/diaminobenzidine/english youglish.com+1
IPA: /ˌdaɪ.əˌmɪn.ə.bɛnˈzɪd.iːn/ *(approximation — long word)*
Phonetic Spelling: dye-uh-min-oh-ben-ZI-deen

- **microliters**

Pronunciation link: https://www.merriam-webster.com/dictionary/microliter Merriam-Webster
IPA: /ˈmaɪkroʊˌli·tər/
Phonetic Spelling: MY-kro-lie-ter

- **humidity**

Pronunciation link: https://www.merriam-webster.com/dictionary/humidity Merriam-Webster
IPA: /hju·ˈmɪd·ə·ti/
Phonetic Spelling: hyu-MID-i-tee

- **compound** *(as in "optimal cutting temperature compound")*

Pronunciation link: https://www.merriam-webster.com/dictionary/compound Merriam-Webster
IPA: /ˈkɑmˌpaʊnd/
Phonetic Spelling: KOM-pound

- **tumor** *(if you have variations like "tumour" or tissues from tumors — though "tissue" was used, but conventionally many protocols use tumor; user text uses "tissue," but "tissue" is straightforward, so not included)*

- **pinhole** *(in microscopy context)*

Pronunciation link: https://www.merriam-webster.com/dictionary/pinhole Merriam-Webster
IPA: /ˈpɪnˌhoʊl/
Phonetic Spelling: PIN-hole

- **optical** *(as in "optical sectioning")*

Pronunciation link: https://www.merriam-webster.com/dictionary/optical Merriam-Webster
IPA: /ˈɑp·tɪ·kəl/
Phonetic Spelling: OP-ti-kul

- **fluorescence**

Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence Merriam-Webster
IPA: /flʊəˈrɛs·əns/
Phonetic Spelling: flu-uh-RESS-ens