FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 68124
Scriptwriter Name: Sulakshana Karkala
Project Page Link: https://review.jove.com/account/file-uploader?src=20777868

Title: DNA-Barcode-Based Multiplex Immunofluorescence Imaging to Analyze FFPE Specimens from Genetically Reprogrammed Murine Melanoma

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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, all done**

3. Filming location: Will the filming need to take place in multiple locations? **Yes, Same building, different floors.**

Current Protocol Length
Number of Steps: 29
Number of Shots: 56

Introduction

Videographer: Obtain headshots for all authors available at the filming location. Also obtain the names of the authors who deliver the introduction statements

1.1. **Joel Sunshine:** We present a protocol for developing and validating a multiplex immunofluorescence panel to study mouse FFPE tissues, and demonstrate its utility by studying samples from a melanoma model treated with nanoparticles delivering plasmid DNA encoding immunologic signals for reprogramming the tumor microenvironment.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.4*

What research gap are you addressing with your protocol?

1.2. **Joel Sunshine:** The field was missing rigorously developed and validated multiplex immunofluorescence protocols compatible with mouse formalin fixed paraffin embedded (FFPE) tissues.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll: 2.3*

What advantage does your protocol offer compared to other techniques?

1.3. **Sachin S. Surwase :** FFPE preserves tissue morphology long-term, is easy to handle and store, and enables creation of TMAs for viewing multiple specimens on a single slide.

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

How will your findings advance research in your field?

1.4. **Xin Ming M. Zhou:** Spatial proteomics imaging will enhance the visualization and analysis of the complex TME, ultimately aiding in the prediction of immune responses, which can contribute to the development of better therapeutics.

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

What research questions will your laboratory focus on in the future?

1.5. **Joel Sunshine:** We will be using this protocol to enable improved spatial profiling of mouse tumor models across many more tumor types and in different immune organs.

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

2. Formalin-Fixed Paraffin-Embedded (FFPE) Tissue Staining and Imaging for Multiplex Analysis

Demonstrator: Sachin S. Surwase

Videographer's Note: File B070_05051346_C026.mov was a false start on the camera and so was omitted from the upload

2.1. To begin, bake the FFPE *(F-F-P-E)* tissue slides at 60 degrees Celsius, overnight **[1-TXT]**. The next day, cool the slides at room temperature for 10 minutes before starting deparaffinization and rehydration **[2]**.

2.1.1. WIDE: Talent placing FFPE tissue slides in a 60°C oven. **TXT: FFPE: Formalin-Fixed Paraffin-Embedded**

2.1.2. Talent removes the slides from the oven and places it on a tabletop.

2.2. Next, incubate the slides in xylene solution twice for 5 minutes each to remove paraffin **[1]**. Then rehydrate the tissue by moving the slides through 100% ethanol, twice, for 5 minutes each **[2]**.

2.2.1. Talent placing slides in xylene for deparaffinization.

2.2.2. Talent transfers the slides to 100% alcohol.

Videographer's Note: Take 2 was better

2.3. Place the slides sequentially in 90%, 70%, 50%, and 30% ethanol solutions for 5 minutes each **[1]**. Wash the slides twice with distilled water for 5 minutes each **[2].** Perform antigen retrieval **[3]** then use fresh bleaching solution to bleach the tissue was twice for 45 min minute intervals **[4]** before performing antibody staining **[5]**.

2.3.1. Talent sequentially immersing slides in different ethanol concentrations.

2.3.2. Talent placing the slides in distilled water.

Added Shot: Talent performing the antigen retrieval step.

Added Shot: Talent performing the tissue bleaching steps twice.

Videographer's Note: Added shots have 2 shots each. First added shot is labelled 2.3.2 a,b and 2nd one is 2.3.2 c,d

2.3.3. Shot of antibody-stained slides.

Videographer's Note: Last take is best

2.4. Next, remove the plastic chamber and store the collected antibody cocktail at 4 degrees Celsius to fix the stain **[1]**. Wash the slides twice in staining buffer for 2 minutes each **[2]**.

2.4.1. Talent removing the plastic chamber and storing antibody cocktail.

2.4.2. Talent placing slides in staining buffer.

2.5. Incubate the slides in a dish containing 40 milliliters of post-staining fixing solution for 10 minutes **[1-TXT]**. Then rinse them with PBS three times for 2 minutes each **[2]**.
2.5.1. Talent transferring slides to post-staining fixation solution. **TXT: Post-Staining Fixing Solution: 4 mL 16% PFA, 36 mL storage buffer**
2.5.2. Talent placing the slides in PBS.

2.6. Now transfer the slides into ice-cold methanol for 5 minutes before rinsing with PBS as before **[1]**. Apply 200 microliters of final fixative solution to each slide under a humidity chamber **[2-TXT]**.
2.6.1. Talent immersing slides in ice-cold methanol.
2.6.2. Talent applying fixative solution to slides. **TXT: Fixative solution: 20 µL/tube + 1 mL of PBS**

2.7. After a 20-minute incubation at room temperature, wash the slides in PBS again, for 3 cycles of 2 minutes each **[1]**.
2.7.1. Talent transfers the slides to PBS.

2.8. If imaging immediately, wipe the slide around the tissue using a lint-free tissue **[1]**. Press the flow cell for 30 seconds using flow cell assembly equipment **[2]**.
2.8.1. Talent wiping slides.
2.8.2. Talent pressing the flow cell on the slide.

2.9. If imaging later, store the slide without applying the flow cell in the storage buffer at 4 degrees Celsius **[1]**. When ready to image, transfer the slides from storage buffer to PBS for 10 minutes before applying the flow cell **[2]**.
2.9.1. Talent places the slide in storage buffer and keeps it at 4 degrees Celsius.
2.9.2. Talent transferring slides from storage buffer to PBS before imaging.

2.10. Next, prepare running and DMSO buffers based on the desired imaging cycles **[1]**. Prepare the reporter stock solution required for the total number of imaging cycles **[2]**.
2.10.1. Talent preparing buffers.
AND
TEXT ON PLAIN BACKGROUND:
Low DMSO (1:4) Buffer: 1-part DMSO with 4-parts running buffer
High DMSO (9:1) Buffer: 9-parts DMSO with 1-part running buffer
Video Editor: Please show both shots side by side
2.10.2. Shot of labelled prepared reporter stock solution.

2.11. Pipette 250 microliters of reporter stock solution and 5 microliters of each reporter into labelled black or amber 1-milliliter microcentrifuge tubes **[1]**.

2.11.1. Talent pipetting reporter stock solutions into each tube.

Added Shot: Talent adding the 5 microliters of reporters into the tubes.

2.12. Transfer the solution to a black 96-well plate and seal it with adhesive foil **[1]**.

2.12.1. Shot of solution being transferred to a black 96-well plate and sealed with adhesive foil.

2.13. Now, start the imaging device and use the instrument manager to set the parameters and exposure times **[1]**. Place the reporter plate in the device **[2]** and then place the slide in the microscope holder to begin imaging **[3].** Acquire images of stained tissues **[4]**.

NOTE: VO has been edited to accommodate the added shots

Added shot: Talent showing setting of exposure times.

AND

2.13.1. SCREEN: 68124_Section2_Screenshot_1.mp4 00:04-00:33

Video Editor: Please show both shots side by side

Added shot: Talent placing the reporter plate in the device

Added shot: Talent placed the slide in the microscope holder and began imaging

2.13.2. SCREEN: 68124_Section2_Screenshot_2.mp4 01:02-01:11

3. Digital Pathology Analysis and Proteomics Data Normalization

Demonstrator: Xin Ming M. Zhou

3.1. Install the latest version of digital pathology analysis software **[1]**. Click **Create Project** and select a destination folder for the project space **[2]**.

3.1.1. Talent installing/launching the latest version of the digital pathology analysis software.

3.1.2. SCREEN: 68124_Section3_Screenshot_1_1080p.mp4 00:00-00:14

3.2. Next, click on **Add images** followed by **Choose files**, then navigate to the QPTIFF *(Q-P-tiff)* file produced from multiplex immunofluorescence imaging **[1]**. Set the image type to **Fluorescence**, keep default settings, and click **Import [2]**.

3.2.1. SCREEN: 68124_Section3_Screenshot_2_1080p.mp4 00:00-00:11

3.2.2. SCREEN: 68124_Section3_Screenshot_2_1080p.mp4 00:17-00:27

3.3. If using QuPath *(Q-path)*, double-click the new image to open a workspace, then press **File** and **Save** periodically to track project changes **[1]**. Toggle marker visibility and viewing settings using the **Brightness & Contrast** tool in the toolbar **[2]**.

3.3.1. SCREEN: 68124_Section3_Screenshot_3_1080p.mp4 00:00-00:11

3.3.2. SCREEN: 68124_Section3_Screenshot_3_1080p.mp4 00:15-00:29

3.4. Use the brush or wand tool to draw an annotation around the entire tissue section **[1]**. Define this annotation as **Full_Tissue** *(Full-tissue)* while excluding overlying skin or regions that should not be analyzed **[2]**. Hold the Alt key to create negative annotations or shrink boundaries **[3]**.
3.4.1. SCREEN: 68124_Section3_Screenshot_4_1080p.mp4. 00:00-00:15
3.4.2. SCREEN: 68124_Section3_Screenshot_4_1080p.mp4. 00:16-00:24
3.4.3. SCREEN: 68124_Section3_Screenshot_4_1080p.mp4. 00:26-00:34

3.5. Now choose the annotation by clicking the **Annotations** tab and pressing **Objects** > followed by **Annotations...** *(Annotations)* then click **Duplicate selected annotations [1]**. Turn on the **SOX10** *(socks-ten)* channel, then shrink the duplicated annotation to define the Tumor region using **Alt + brush/wand** *(Alt-plus-Brush-wand)* tool **[2]**.
3.5.1. SCREEN: 68124_Section3_Screenshot_5_1080p.mp4. 00:00-00:08
3.5.2. SCREEN: 68124_Section3_Screenshot_5_1080p.mp4. 00:18-00:24, 00:27-00:48

3.6. Now, select the Full_Tissue annotation, then go to **Objects** followed by **Annotations...** and **Expand annotations [1]**. Set the **Expansion radius** to 1 micrometer and click **Run [2]**.
3.6.1. SCREEN: 68124_Section3_Screenshot_6_1080p.mp4. 00:00-00:07
3.6.2. SCREEN: 68124_Section3_Screenshot_6_1080p.mp4. 00:08-00:14

3.7. Rename this new annotation as Full_Tissue_Expansion **[1]**. Select the **Tumor Annotation**, right-click, and select **Insert in hierarchy [2]**.
3.7.1. SCREEN: 68124_Section3_Screenshot_7_1080p.mp4. 00:10-00:16
3.7.2. SCREEN: 68124_Section3_Screenshot_7_1080p.mp4. 00:16-00:22

3.8. Re-select the **Tumor annotation**, then go to **Objects** , **Annotations...** and now click **Make inverse [1]**. Define this new annotation as Stroma and repeat for all tissue sections **[2]**.
3.8.1. SCREEN: 68124_Section3_Screenshot_8_1080p.mp4. 00:00-00:08
3.8.2. SCREEN: 68124_Section3_Screenshot_8_1080p.mp4. 00:09-00:20

3.9. To run cell segmentation, click on the **Image** tab to find the pixel width and image height **[1]**.
3.9.1. SCREEN: 68124_Section3_Screenshot_9_1080p.mp4 00:00-00:12.

3.10. Download the StarDist *(Star-Dist)* extension from GitHub *(Git-hub)* **[1]**. Import the qupath-extension-stardist-[version].jar *(Q-path-extension-stardist-version-dot-jar)* file

into QuPath **[2]**. Also download the StarDist groovy script files and model file from GitHub **[3]**.

3.10.1. SCREEN: 68124_Section3_Screenshot_10_1080p.mp4. 00:00-00:13
3.10.2. SCREEN: 68124_Section3_Screenshot_10_1080p.mp4. 00:14-00:24
3.10.3. SCREEN: 68124_Section3_Screenshot_10_1080p.mp4. 00:25-00:47

3.11. For each tissue section, select both **Tumor** and **Stroma annotations [1]**. In the settings bar, click **Automate** followed by **Script Editor** to open the script interface **[2]**. Open the appropriate StarDist cell segmentation script corresponding to the image pixel size **[3]**. When prompted, select stardist_cell_seg_model.pb *(stardist-cell-seg-model-dot-P-B)* for cell segmentation **[4]**.

3.11.1. SCREEN: 68124_Section3_Screenshot_11_1080p.mp4 00:00-00:05.
3.11.2. SCREEN: 68124_Section3_Screenshot_11_1080p.mp4 00:06-00:10.
3.11.3. SCREEN: 68124_Section3_Screenshot_11_1080p.mp4 00:11-00:20
3.11.4. SCREEN: 68124_Section3_Screenshot_11_1080p.mp4 00:25-00:34.

3.12. After cell segmentation, save the image. Then sequentially click on **Measure**, **Export Measurements**, select the **Corresponding image(s)**, and set **Export type** as **Cells** and **Separator** as **.csv** *(Dot-C-S-V)* **[1]**. Choose an output file location, then click **Populate** to include relevant metrics **[2]**.

3.12.1. SCREEN: 68124_Section3_Screenshot_12_1080p.mp4. 00:00-00:21
3.12.2. SCREEN: 68124_Section3_Screenshot_12_1080p.mp4. 00:22-00:32

3.13. For each lineage marker to be used in clustering or phenotyping, select one mean value to export **[1-TXT].**

3.13.1. SCREEN: 68124_Section3_Screenshot_13_1080p.mp4 00:00-00:26

TXT: Export Nucleus: Mean for nuclear markers and Cytoplasm:Mean for cytoplasmic markers

3.14. For normalization of proteomic data, open the exported CSV file and truncate the column headings to retain only the marker name **[1].**

3.14.1. SCREEN: 68124_Section3_Screenshot_14_1080p.mp4 00:00-00:18

3.15. After installing the latest version of R and RStudio *(R-Studio)*, download the Marker Normalization.R *(Marker-Normalization-dot-R)* script from GitHub and run it to filter out cells based on nuclear size **[1]**.

3.15.1. SCREEN: 68124_Section3_Screenshot_15_1080p.mp4 00:00-00:27

3.16. Perform minimum-maximum normalization for each marker **[1-TXT]**. ~~Set the lowest Mean Fluorescence Intensity or MFI *(M-F-I)* value in the range to 0 and the 99.7th~~

~~percentile MFI value to 1 **[2].** Clip any intensities above the 99.7th percentile to 1 **[2].**~~
Once complete, save the new CSV file with the normalized data **[3]**.

3.16.1. SCREEN: 68124_Section3_Screenshot_15_1080p.mp4 00:02-00:20 **TXT: Set lowest MFI to 0 and 99.7th percentile to 1; Clip intensities above 99.7th percentile to 1**

3.16.2. SCREEN: 68124_Section3_Screenshot_15_1080p.mp4 00:21-00:34

Results

4. Representative Results

4.1. Antibody-DNA barcode conjugation was confirmed by protein gel electrophoresis, showing additional bands at the heavy chain region **[1]**.

4.1.1. LAB MEDIA: Figure 4. *Video editor: please highlight the "Heavy chain" band*

4.2. Multiplex immunofluorescence imaging of B16F10 *(B-sixteen-F-ten)* flank tumors treated with 4-1BBL/IL-12 *(four-one-B-B-L-I-L-twelve)* nanoparticles and systemic anti-PD1 *(Anti-P-D-one)* showed distinct immune marker expression across tumor sections **[1]**.

4.2.1. LAB MEDIA: Figure 5.

4.3. FlowSOM *(flow-some)* clustering identified a wider range of marker expression intensities compared to Seurat phenotyping, with a maximum intensity difference of approximately 0.7 versus 0.5 **[1]**. FlowSOM phenotyping also classified a higher number of macrophages into M1 and M2 subtypes **[2]**.

4.3.1. LAB MEDIA: Figure 6A and B. *Video editor: please show B first then A*

4.3.2. LAB MEDIA: Figure 7. *Video editor: please highlight the purple areas of the FlowSOM phenotyping image*

4.4. Quantification of macrophage densities showed that FlowSOM detected higher densities of both M1 and M2 macrophages compared to Seurat **[1]**, while Seurat classified more macrophages as "other" **[2]**.

4.4.1. LAB MEDIA: Figure 8A-B. *Video editor: please highlight the blue columns*

4.4.2. LAB MEDIA: Figure 8C *Video editor: please highlight the red column*

4.5. Spatial analysis revealed that M2 macrophages and natural killer cells had the highest average minimum distances following treatment **[1]**, and M1 macrophages were more prevalent around CD8 *(C-D-Eight)* T cells **[2]**.

4.5.1. LAB MEDIA: Figure 9A. *Video editor: Emphasize the columns of Target Cell Type corresponding to M2 and NK (blue columns)*

4.5.2. LAB MEDIA: Figure 9B *Video editor: Please highlight the CD8-M1 row*

Pronunciation Guide:

1. **Immunofluorescence**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/immunofluorescence
   IPA: /ˌɪmjənoʊflʊˈrɛsəns/
   Phonetic Spelling: ih-myoo-noh-floo-REH-sns
2. **Paraffin**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/paraffin
   IPA: /ˈpærəfɪn/
   Phonetic Spelling: PAIR-uh-fin
3. **Murine**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/murine
   IPA: /ˈmjʊraɪn/
   Phonetic Spelling: MYUR-ine
4. **Melanoma**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/melanoma
   IPA: /ˌmɛləˈnoʊmə/
   Phonetic Spelling: mel-uh-NOH-muh
5. **QPTIFF**
   Pronunciation link: No confirmed link found
   IPA: /kjuː-piː-tɪf/
   Phonetic Spelling: KYOO-pee-tiff
6. **QuPath**
   Pronunciation link: No confirmed link found
   IPA: /ˈkjuːpæθ/
   Phonetic Spelling: KYOO-path
7. **SOX10**
   Pronunciation link: No confirmed link found
   IPA: /sɑks tɛn/
   Phonetic Spelling: SOCKS-ten
8. **Stroma**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/stroma
   IPA: /ˈstroʊmə/
   Phonetic Spelling: STROH-muh
9. **StarDist**
   Pronunciation link: No confirmed link found
   IPA: /stɑr-dɪst/
   Phonetic Spelling: STAR-dist

10. **Proteomics**
    Pronunciation link:
    https://www.merriam-webster.com/dictionary/proteomics
    IPA: /ˌproʊtiˈɑmɪks/
    Phonetic Spelling: proh-tee-AH-miks
11. **Electrophoresis**
    Pronunciation link:
    https://www.merriam-webster.com/dictionary/electrophoresis
    IPA: /ɪˌlɛktrəfəˈrisɪs/
    Phonetic Spelling: ih-lek-troh-fuh-REE-sis
12. **FlowSOM**
    Pronunciation link: No confirmed link found
    IPA: /floʊ-sɑm/
    Phonetic Spelling: FLOH-sahm
13. **Seurat**
    Pronunciation link: No confirmed link found
    IPA: /ˈsɜːrɑː/ (based on bioinformatics usage; differs from French painter /sɜːˈrɑː/)
    Phonetic Spelling: SUR-rah
14. **Macrophages**
    Pronunciation link:
    https://www.merriam-webster.com/dictionary/macrophage
    IPA: /ˈmækrəˌfeɪdʒ/
    Phonetic Spelling: MAK-roh-fayj
15. **CD8**
    Pronunciation link: No confirmed link found
    IPA: /ˌsiːˌdiːˈeɪt/
    Phonetic Spelling: SEE-DEE-eight
16. **Nanoparticles**
    Pronunciation link:
    https://www.merriam-webster.com/dictionary/nanoparticle
    IPA: /ˈnænoʊˌpɑrtɪkəlz/
    Phonetic Spelling: NAN-oh-par-ti-klz
17. **Plasmid**
    Pronunciation link:
    https://www.merriam-webster.com/dictionary/plasmid
    IPA: /ˈplæzmɪd/
    Phonetic Spelling: PLAZ-mid
18. **Tissue Microarray (TMA)**
    Pronunciation link (Tissue):
    https://www.merriam-webster.com/dictionary/tissue
    Pronunciation link (Microarray):
    https://www.howtopronounce.com/microarray

IPA: /ˈtɪʃuː ˈmaɪkroʊəˌreɪ/
Phonetic Spelling: TISH-oo MY-kroh-uh-ray

19. **Antigen**
Pronunciation link:
https://www.merriam-webster.com/dictionary/antigen
IPA: /ˈæntɪdʒən/
Phonetic Spelling: AN-tih-jen